CAUSES AND CONSEQUENCES OF INTRAUTERINE GROWTH RESTRICTION

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PUBLISHED IN: Frontiers in Endocrinology and Frontiers in Physiology

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-768-3
DOI 10.3389/978-2-88963-768-3

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CAUSES AND CONSEQUENCES OF INTRAUTERINE GROWTH RESTRICTION

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Citation: Winterhager, E., Bendix, I., Miller, S. L., eds. (2020). Causes and Consequences of Intrauterine Growth Restriction. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-768-3

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frontiers in Endocrinology

EDITORIAL
published: 15 April 2020
doi: 10.3389/fendo.2020.00205

Editorial: Causes and Consequences of Intrauterine Growth Restriction

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Keywords: intrauterine growth restriction, fetal growth restriction, IUGR, FGR, pathology, placenta, brain injury

Editorial on the Research Topic

Causes and Consequences of Intrauterine Growth Restriction

The origins of health and disease can be programmed during prenatal development caused by an unfavorable intrauterine environment, known as "fetal programming." Intrauterine or fetal growth restriction (IUGR/FGR) describes a pathological condition in which the fetus fails to grow to its biological potential, primarily because of poor placental function. In turn, FGR is known to lead to short- and long-term consequences, like cardiovascular, renal, immunological and neurological disease that greatly impact on individuals and society. Adequate diagnostic approaches and therapeutic interventions are lacking. Thus, dissecting cellular and molecular mechanisms causing placental dysfunction, suboptimal fetal/offspring organ development, and disturbed genetic/epigenetic programming in the offspring are great challenges all required so that interventions can be explored. Defining individual causes for FGR will enable a better diagnosis and raise possible translational candidates for intervention to improve the health of the FGR offspring.

The aim of the present collection "Causes and Consequences of Intrauterine Growth Restriction (IUGR)" was to bring together the many causes for fetal growth restriction, and to describe the consequences of predisposition toward various diseases in later life. The 21 contributions, both reviews and original research papers, include basic science and clinical studies to highlight the placental origins of growth restriction, difficulties associated with *in utero* diagnosis, possible mechanisms initiating an adverse maternal environment, together with the consequences of fetal programming for neonatal and life-long health, plus discussion on potential interventions.

In putting together this collection of studies we became even more aware how diverse this problem is, and that we remain far away from identifying molecular mechanisms in common, or to ascertain a simple concept about the origin of health and disease. After a short discussion with Salavati et al., one of the contributing authors, the guest editors of this collection would like to shift the term intrauterine growth retardation/restriction (IUGR), toward the term fetal growth restriction (FGR). As investigators in the field, we agreed that the term FGR more appropriately addresses that it is the individual rather than the environment that is at risk. Furthermore, we would like to encourage a shift away from using the terms small for gestational age (SGA) and FGR interchangeably—in past years both terms have described an estimated fetal weight/ birth weight <10th centile, but SGA should not be used as a synonym for FGR. Most importantly, the difference between SGA and FGR refers to the fact that FGR fetuses are pathologically growth-restricted and demonstrate a reduction in growth trajectory as placental function fails. SGA describes a fetus that is small, but with a normal growth trajectory and normal umbilical artery Doppler velocimetry that indicate the SGA fetus is growing constitutively small along its genetically determined size. In contrast, FGR fetuses do not reach their genetically determined potential size due a pathological interruption to normal growth, most often resulting from placental insufficiency. Thus, SGA

OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *20 February 2020*
Accepted: *24 March 2020*
Published: *15 April 2020*

Citation:
Bendix I, Miller SL and Winterhager E (2020) Editorial: Causes and Consequences of Intrauterine Growth Restriction. Front. Endocrinol. 11:205. doi: 10.3389/fendo.2020.00205

defines a statistical deviation only of size but not the pathological condition. Moreover, it would be worthwhile to address a subdivision of early-onset FGR (<32 weeks), and late-onset FGR (≥32 weeks) in future investigations because the two different time intervals are probably due to different causes of placental dysfunction and certainly result in varied consequences for the growth restricted infant.

Besides genetic defects or intrauterine infections, FGR is primarily attributed to a dysfunctional placenta. Lessons from mutant mice demonstrate that defective placental structures are clearly related to FGR. The review of Woods et al. summarizes studies of common types of placental defects in established mouse mutants, which help to instruct us in gaining a better understanding of placental gene expression pattern with an impact on failures in human placentation. In addition, gene expression responsible for the development of the different placental structures is often modulated by epigenetic mechanisms.

Identifying impairment of placental development is another challenge; Salavati et al. show that screening of defective human placental development by morphometry could serve to identify FGR at early stages for a better clinical management.

Placental dysfunction and reduced nutrient transfer to the fetus is a primary mediator of reduced fetal growth, however the placenta may be able to compensate and thus sustain the health of the developing fetus. Using mouse mutants defective in IGF2, Hayward et al. demonstrated that the placenta is able to compensate for the calcium transport by a decrease in calcium binding proteins, the parathyroid hormone-related protein and an increase in serum/glucocorticoid-regulated kinase. Another compensating mechanism is described by Schmidt et al. using a FGR rat model fed with low protein diet during pregnancy, which revealed that offspring showed significantly reduced levels of local corticosterone, probably a mechanism to sustain brain development. Endogenous compensation mechanisms are rarely investigated within FGR research but could help to identify novel treatment options in future. Placental infection during pregnancy is also a main cause FGR, and the review of Seitz et al. describes the molecular mechanisms of Plasmodium falciparum infection—still an important issue and reason for FGR in tropical/ subtropical regions—via a defined surface antigen. Deciphering such infection strategies might give rise to intelligent treatment options.

The pregnancy disease preeclampsia is a primary complication associated with FGR, and with an increase in the anti-angiogenic soluble fms-like tyrosine kinase 1 (sFlt-1) of the placenta. Vogtmann et al. presents an hs-Flt-1 overexpressing mouse model which evidenced that sFlt-1 is responsible for the development of FGR because of a reduced placental efficiency with changes in the maternal/fetal vasculature.

Several studies highlighted the often neglected maternal impact of FGR. A multicentre trial by Feenstra et al. revealed that maternal vascular malperfusion contributes to FGR in late pregnancy. Maternal vascularization at the feto-maternal unit in humans and mice involves uterine vasodilation and vessel remodeling upon trophoblast invasion with the help of immune cells. Using a specific deletion of the progesterone receptor in mouse dendritic cell, Thiele et al. showed that impairment of the cross talk between progesterone and dendritic cells leads to a reduction of pregnancy-protective immune cells leading to a poor placentation and FGR. These findings are corroborated by studies on pregnant women from Dunk et al., where defective progesterone-mediated poor decidualization is associated with an elevated mature dendritic profile and the failure of utero-vascular remodeling in FGR.

This review opens discussion about functional connections between placental malformation/malfunction and cardiovascular as well as brain diseases in adults. At this point it needs to be mentioned that not only FGR infants could develop a cardiovascular disease later in life, but there are long term cardiovascular consequences for mothers who develop preeclampsia during pregnancy, a topic which is very interesting however, not highlighted in our collection.

Langmia et al. summarize the effects of prolonged adverse maternal environment such as diabetic pregnancies as well as undernutrition and hypoxic conditions on placental genetic/epigenetic modifications, which in turn contribute to FGR combined with cardiovascular programming in rodents and humans. Malhotra et al. turn attention to consequences of growth restriction for the neonate after birth, and brings together a review of literature to show that FGR is associated with respiratory, cardiovascular, and neurological morbidities after birth that may require extra management in neonatal intensive care. The presence and severity of neonatal and longer term respiratory, cardiovascular, and neurological deficits reflect whether FGR is early- or late-onset, the severity of growth restriction, the degree of fetal cardiovascular adaptation, and the gestation at birth. Neonatal pathology is substantiated by Villamor-Martinez et al. who show that FGR but not SGA babies maintain a patent ductus arteriosus after birth which again emphasizes the importance to discriminate between SGA and FGR in clinical studies. The pulmonary consequences of FGR are examined by Allison et al. in FGR and appropriately-grown preterm lambs, together with the potential therapeutic effects of early intervention with umbilical cord blood stem cells.

Obstetric management can improve catch-up growth of FGR babies and also neurodevelopmental and behavioral outcomes (van Wyk et al.). When growth restriction is evoked by calorie restricted diet during rat pregnancy, intervention with lactoferrin ameliorates FGR, and has a neuroprotective effect as shown by van de Looij et al. Clinical investigations for health consequences of the offspring need to carefully discriminate between babies only with FGR, with FGR and premature birth, and babies defined as SGA babies. In this context Morsing et al. could demonstrate that reduced brain volumes as determined by MRI, normally judged as a consequence for FGR, are more likely due to premature birth.

Many studies deal with the consequences of an adverse maternal environment for brain development in growth restricted offspring. A good deal of those maternal insults summarized by Baud and Berkane include dysregulation of steroid hormones and growth factors that mediate placental feto-maternal exchange for fetal growth and neurodevelopment. The longer term neurological consequences of placental

insufficiency and FGR are increasingly well-understood, particularly as standardized definitions of FGR and SGA are used, together with standardized assessment tools (Vollmer and Edmonds). Hartkopf et al. examined the origins of altered neurodevelopmental function in FGR offspring, and observed that fetal responses show early evidence of altered brain development, while at 2 years of age, children born FGR were found to have neurodevelopmental delays. Indeed the review by Vollmer and Edmonds describes that school-age children who were born growth restricted are more likely to demonstrate cognitive, behavioral, and/or attention deficits and this is particularly so when FGR and preterm birth are co-morbidities.

The review of Fleiss et al. summarizes research and clinical efforts to identify known pathogenic mechanisms that contribute to FGR and, in particular, neurodevelopmental compromise, and highlight knowledge gaps that require addressing. Quite sensibly, they demand a more concerted research focus and global networking on this complex topic. Yawno et al. examined the ontogeny of cerebellar (mal-)development in the final trimester of fetal sheep FGR and showed that altered brain cerebellar growth is evident antenatally, and continued *in utero* compromise leads to progressive worsening in development of the cerebellum. Together, these preclinical and clinical studies provide good evidence that altered brain development begins *in utero* due to placental compromise, and therefore early diagnosis and early intervention to improve neurological function in FGR offspring are key research areas.

CONCLUSION

The collection of articles presented here in this special issue aimed to describe the various causes and many of the consequences for a baby developed in an adverse intrauterine environment wherein placental insufficiency leads to fetal growth restriction. We described the maternal insults or pregnancy compromise that may seriously affect fetal growth and well-being of the offspring including pregnancy diseases, eating disorders, infection/inflammation, and hormonal imbalance. It is however apparent that fetal growth restriction is primarily caused by placental dysfunction, leading to chronic fetal hypoxia and hypoglycaemia. It is these consequences of placental insufficiency that lead to reduced fetal growth overall, and suboptimal organ development. FGR is associated with lifelong burden of chronic diseases including metabolic, respiratory, cardiovascular and neurological deficits that greatly impact on individuals and society. At this time it remains that adequate diagnostic approaches as well as therapeutic interventions are lacking. The many studies in animals as well as in clinical trials reveal how complex and difficult it is to define a clear phenotype, and as a consequence, to implement an appropriate management strategy during pregnancy, a period which has high ethical limitations in treatment. Thus, in this topic we have described that FGR is strongly linked to altered neonatal and long-term outcomes, and research to improve outcomes must be based on an improved understanding of the pathophysiological mechanisms that alter organ development and growth. Herein our author contributors propose better collaborative research networks that will continue working toward standardized diagnosis and outcome assessments, and will continue research to increase our understanding of the complex changes that occur due to placental insufficiency, so that improved clinical management and treatment is implemented to reduce the burden of FGR.

AUTHOR CONTRIBUTIONS

All authors contributed as editors of this Research Topic, and wrote and edited this editorial.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 19 September 2018
doi: 10.3389/fphys.2018.01278

Impact of Intrauterine Growth Restriction on Cognitive and Motor Development at 2 Years of Age

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OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *17 May 2018*
Accepted: *22 August 2018*
Published: *19 September 2018*

Citation:
Hartkopf J, Schleger F, Keune J, Wiechers C, Pauluschke-Froehlich J, Weiss M, Conzelmann A, Brucker S, Preissl H and Kiefer-Schmidt I (2018) Impact of Intrauterine Growth Restriction on Cognitive and Motor Development at 2 Years of Age. Front. Physiol. 9:1278. doi: 10.3389/fphys.2018.01278

Intrauterine growth restriction (IUGR), which is already known to be a risk factor for pathological intrauterine development, perinatal mortality, and morbidity, is now also assumed to cause both physical and cognitive alterations in later child development. In the current study, effects of IUGR on infantile brain function were investigated during the fetal period and in a follow-up developmental assessment during early childhood. During the fetal period, visual and auditory event-related responses (VER and AER) were recorded using fetal magnetoencephalography (fMEG). VER latencies were analyzed in 73 fetuses (14 IUGR fetuses) while AER latencies were analyzed in 66 fetuses (11 IUGR fetuses). Bayley Scales of Infant Development, Second Edition (BSID-II) were used to assess the developmental status of the infants at the age of 24 months. The Mental Development Index (MDI) was available from 66 children (8 IUGR fetuses) and the Psychomotor Development Index (PDI) from 63 children (7 IUGR fetuses). Latencies to visual stimulation were more delayed in IUGR than in small for gestational age (SGA) or appropriate for gestational age (AGA) fetuses, albeit not to any significant extent (p = 0.282). The MDI in former IUGR infants was significantly lower (p = 0.044) than in former SGA and AGA infants. However, IUGR had no impact on PDI (p = 0.213). These findings support the hypothesis that IUGR may constitute a risk factor for neurodevelopmental delay. Further investigation of the possible underlying mechanisms, as well as continued long-term developmental research, is therefore necessary.

Keywords: intrauterine growth restriction, child development, fetal magnetoencephalography, visual event-related responses (VER), auditory event-related responses (AER)

Abbreviations: AER, auditory event-related responses; AGA, appropriate for gestational age; BSID-II, Bayley Scales of Infant Development, Second Edition; BSID-III, Bayley Scales of Infant and Toddler Development, Third Edition; fMEG, fetal magnetoencephalography; fT, femto Tesla; GA, gestational age; Hz, Hertz; ISI, inter-stimulus interval; IUGR, intrauterine growth restriction; MDI, Mental Development Index; ms, millisecond; PDI, Psychomotor Development Index; SGA, small for gestational age; VER, visual event-related responses.

INTRODUCTION

Over the last few decades, it has become evident that events during early development in humans – even during the prenatal phase – can have long-term effects on health and disease. This concept is commonly known as Developmental Origins of Health and Disease (Barker, 2007; Wadhwa et al., 2009).

One trademark of anomalous prenatal development is intrauterine growth restriction (IUGR). Intrauterine growth restriction is characterized by a pathological restriction of fetal weight, as is presumed to be the case when a fetus is "small for gestational age" (SGA), i.e., when its estimated fetal weight and birth weight are below the 10th percentile for gestational age (GA). The literature and practice often does not distinguish clearly between IUGR and SGA. Consistent criteria are therefore required to establish general valid guidelines in diagnosis and treatment (Barker et al., 2013; Unterscheider et al., 2014; Levine et al., 2015). SGA, which is a more general term for those fetuses and infants whose estimated and actual birth weights are below the 10th percentile, is not necessarily connected with a pathological finding. The term also includes cases of below-average weight caused by genetic preconditions. By contrast, IUGR is associated with pathological intrauterine changes that cause restricted fetal growth. It is also linked to a higher risk of perinatal mortality and morbidity and requires appropriate medical support (Craigo, 1994; Bamberg and Kalache, 2004). It is important to distinguish between pathologically growth-restricted fetuses and constitutionally small fetuses. Placental insufficiency, the most frequently observed pathological cause for restricted fetal growth, should be diagnosed by the umbilical artery Doppler velocity (Figueras and Gardosi, 2011). Placental insufficiency is associated with metabolic and hormonal influences on the fetuses and manifests itself by reduced fetal growth and weight gain during pregnancy. These processes can lead to specific alterations in later physical and cognitive development known as "fetal programming" (Godfrey and Barker, 2001; Martin-Gronert and Ozanne, 2012). Since this influence begins during pregnancy, an early investigational approach is advisable.

Recent follow-up studies with former IUGR infants often used only reduced body size or abnormal Doppler for diagnosis of IUGR. A review by Murray et al. (2015) showed that only a small number of studies on the neurodevelopmental outcome in children with IUGR born at 35 weeks of gestation or later used both abnormal Doppler and small size as diagnostic criteria. The authors reported that IUGR is associated with an increased risk for neurodevelopmental delay. Children with fetal circulatory redistribution (i.e., a pathological Doppler) were reported to be more severely affected.

Neurodevelopmental impairments in IUGR infants are reflected by morphological and structural brain alterations and impaired brain function even in utero (D'Hooghe and Odendaal, 1991; Vindla et al., 1997; Nijhuis et al., 2000; Tolsa et al., 2004; Dubois et al., 2008; Lodygensky et al., 2008). In earlier trials, changes in body movements and heart rate were the two main indicators for stimulus processing for investigating the influence of IUGR on functional brain development in utero. Following acoustic or vibroacoustic stimulation, heart rate responses in IUGR fetuses were delayed and their body movement patterns lower than in controls (Gagnon et al., 1988, 1989; Kisilevsky et al., 2014).

Fetal magnetoencephalography (fMEG) is a non-invasive method for measuring fetal brain activity. From the GA of 28 weeks onward, fetal auditory event-related brain responses (AER) and visual event-related brain responses (VER) can be recorded and a decrease of latency can be assumed to be a marker of the maturation and integrity of functional fetal brain development (Schleussner et al., 2001; Eswaran et al., 2002; Schleussner and Schneider, 2004; Holst et al., 2005; Kiefer et al., 2008). Against this background, by demonstrating that IUGR fetuses have slower VER than their appropriate for gestational age (AGA) control counterparts, we recently ascertained that VER latency is associated with fetal outcome (Morin et al., 2015). A follow-up study to determine the impact of VER latencies on early childhood development, i.e., from birth to 24 months of age, is currently under way.

In the present study, we aimed to determine whether fetal outcome affects early childhood development. This entailed a developmental assessment using BSID-II that was performed at the age of 24 months in former IUGR, SGA, and AGA children. Furthermore, we investigated whether VER and AER latencies, as assessed by fMEG, differed between the fetal outcome groups.

MATERIALS AND METHODS

Participants

One hundred and seven women with singleton pregnancies were recruited by the Department of Obstetrics and Gynecology at the University Hospital, Tuebingen. They gave written informed consent of their and their infant's participation prior to the study, which was approved by the local Ethical Committee of the Medical Faculty of the University of Tuebingen (No. 476/2008MPG1). The study was performed in accordance with the relevant guidelines and regulations.

Fifteen of the infants had birth weights below the 10th percentile, and an increased umbilical artery pulsatility index above the 90th percentile for the respective GA was observed during pregnancy. These 15 fetuses were classified as IUGR due to an insufficient placental blood supply. Although 32 of the infants were born with weights below the 10th percentile, they had a normal umbilical artery Doppler during pregnancy and no placental insufficiency was found. These 32 fetuses were classified as constitutionally SGA. Sixty healthy children with an AGA birth weight were included as controls.

fMEG Measurement

To investigate potential differences in brain development already during pregnancy, all participants underwent an fMEG measurement with visual and auditory stimulation to record event-related brain responses of the fetuses from 28 weeks of GA. The fMEG measurement was performed with a magnetoencephalographic system for fetal and neonatal studies (SARA II: SQUID Array for Reproductive Assessment,

VSM MedTech Ltd., Port Coquitlam, BC, Canada). During the measurement, the woman placed her abdomen in an ergonomically shaped array containing 156 primary and 29 reference sensors. Visual stimuli were presented during 10 min of the measurement and consisted of light flashes delivered by fiber optic wire to an LED-light pad that was placed on the maternal abdomen near the location of a fetal eye, as determined via ultrasound. The light flashes had a wavelength of 625 nm and an intensity of 8000 lux; stimulus duration was 500 ms and the ISI was set at random between 1.5 and 2.5 s (Morin et al., 2015).

Auditory stimulation consisted of an oddball-paradigm with pure tones and was presented for a further 10 min of the measurement. Stimulus duration was 500 ms and the ISI was randomly selected between 1900 and 2100 ms. Standard tones, presented with a frequency of 500 Hz, were interspersed with deviant tones presented at 750 Hz to avoid habituation to the standard tone. Stimuli were delivered into a balloon via an air-filled tube placed on the maternal abdomen. The sequence of visual and auditory stimulation was randomized over subjects. Fetal data were recorded with a sampling rate of 610.352 Hz (Muenssinger et al., 2013).

fMEG Data Analysis

Recorded fetal auditory and visual datasets were filtered offline with a high-pass filter of 0.5 Hz and were transformed by a first-order gradiometer to eliminate any external interference. Maternal and fetal heart signals were attenuated by signal space projection (McCubbin et al., 2006). The data was cut into segments ranging from 200 ms before to 1000 ms after the stimulus. A 10 Hz low-pass filter was applied and the average of the segments was calculated. VER and AER were analyzed by visual examination and defined as a peak in a time window of 80–500 ms after the stimulus, with a minimal amplitude of 4 fT in at least four sensors around the fetal head coil. The latency between stimulus onset and peak was documented for further statistical analysis.

Developmental Test

Two years after fMEG measurement, all families were invited to participate in an assessment of their child's development with BSID-II. Of a total of 107 participants, 66 returned for the follow-up assessment. The 41 participants who discontinued were distributed as follows: IUGR group: 7 of 15 children (46.7%), SGA group: 14 of 32 children (43.8%) and AGA group: 20 of 40 children (50%). The most common reasons for non-participation are summarized in **Figure 1**.

The BSID-II was developed for the measurement of the current developmental state of infants and children between 1 and 42 months of age (Nellis and Gridley, 1994). An experienced and trained psychologist, who was unaware of the medical history of the infant, conducted the test with the child in the presence of a parent. The BSID-II is divided into two scales: the MDI and PDI. The cognitive and psychomotor development of a child can therefore be assessed separately. MDI and PDI both have a mean of 100 and a standard deviation (SD) of 15.

Statistics

Data was described as mean ± SD. A preliminary assumption check revealed that data was normally distributed, as assessed by Shapiro–Wilk test ($p > 0.05$), and that there were no univariate or multivariate outliers, as assessed by boxplot and Mahalanobis distance ($p > 0.001$), respectively. MDI, PDI, VER latency, and AER latency were analyzed for differences between fetal outcome groups (IUGR, SGA, and AGA) using one-way ANOVA and Welch's test of unequal variances, respectively. *Post hoc* analyses were performed using the Games-Howell correction method. PASW Statistics 21 (SPSS Inc., Chicago, IL, United States) was used for statistical analysis and the significance level was set to $p < 0.05$.

RESULTS

Auditory event-related responses and VER latencies were measured using fMEG in 107 fetuses. Of these, 15 were IUGR fetuses, 32 were SGA fetuses and 60 were AGA fetuses. **Table 1** shows mean and SD for GA at birth and birth weight. VER latencies could be analyzed in a total of 73 fetuses (14 IUGR, 22 SGA, and 37 AGA) at a mean GA of 34.1 weeks. AER latencies were detectable in a total of 66 fetuses (11 IUGR, 22 SGA, and 33 AGA) at a mean GA of 34.0 weeks.

Mental Development Index and PDI were assessed using BSID-II at a mean (± SD) age of 24.10 (± 0.79) months. MDI was assessed in 66 children (8 IUGR, 18 SGA, and 40 AGA) and was 96 (± 6), 100 (± 16), and 103 (± 13), respectively. PDI was assessed in 63 children (7 IUGR, 16 SGA, and 40 AGA) and was 94 (± 7), 96 (± 11), and 100 (± 10), respectively. Results are presented as box plots in **Figure 2**.

Table 2 shows the results of the univariate one-way ANOVA in a comparison of MDI, PDI, VER latency, and AER latency in the IUGR, SGA, and AGA groups. There were no statistically significant differences between PDI ($p = 0.213$), VER latency ($p = 0.282$), and AER latency ($p = 0.206$). However, the MDI differed significantly between groups ($p = 0.044$) and increased from the IUGR (96 ± 6) to the SGA (100 ± 16) as well as to the AGA group (103 ± 13). Games-Howell *post hoc* analysis (**Table 3**) revealed that the difference between IUGR and AGA was statistically significant ($p = 0.035$).

DISCUSSION

In the current study, we aimed to investigate the impact of IUGR on early child development. At the age of two, children's developmental status was assessed using BSID-II. The MDI was significantly lower in the IUGR than in the AGA group. Although scores for the PDI decreased from AGA to SGA, and IUGR, these differences were not statistically significant. In addition, fetal brain responses to visual and sound stimulation were assessed via fMEG before birth. We observed an increase in VER latencies from AGA over SGA to IUGR fetuses. These latency differences were, however, not statistically significant. Our results suggest that functional brain development maybe

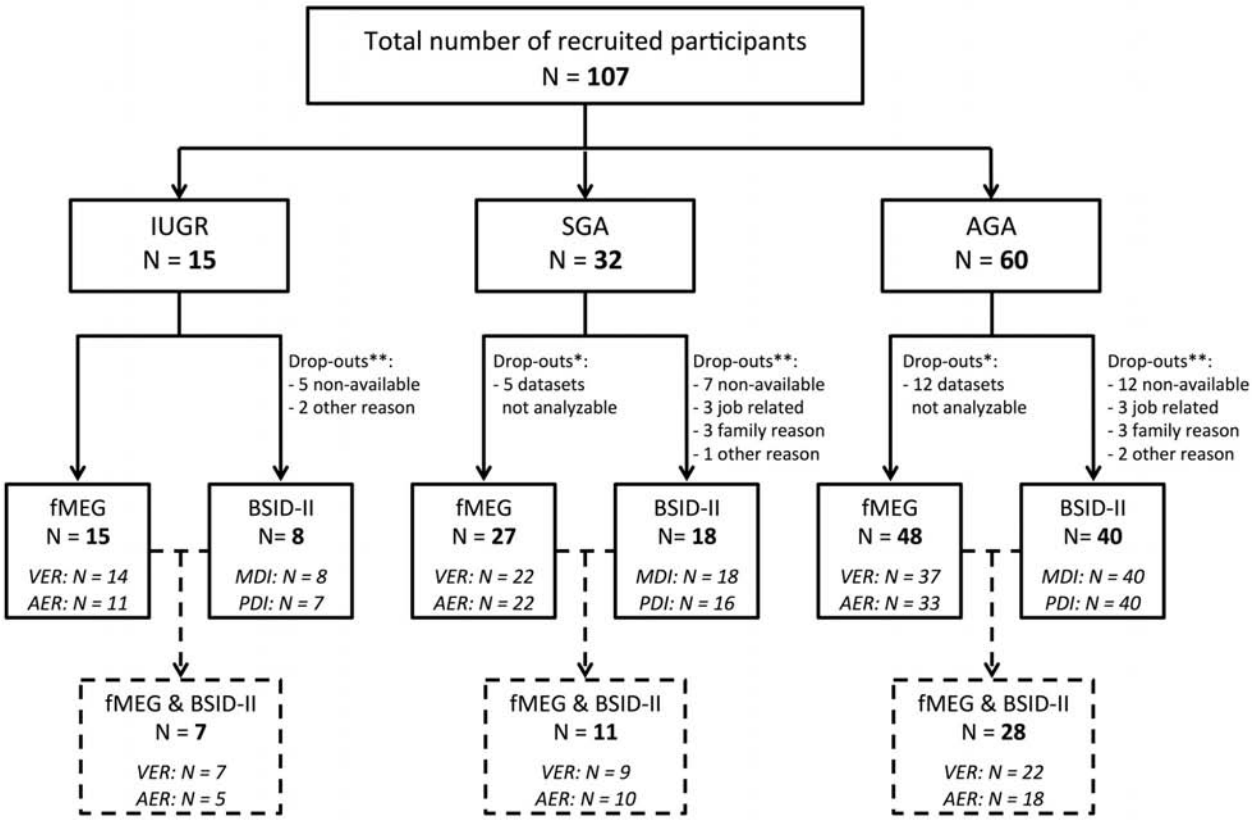

FIGURE 1 | Flow chart showing enrollment and follow-up. All 107 infants were evaluated using fMEG. At the age of 2 years, developmental assessment was performed using BSID-II. *Indicates the number of fMEG data sets that were not analyzable. **Indicates the number of children that were lost to follow-up. At the bottom of the figure, the number of children for whom BSID-II and fMEG was available is shown.

TABLE 1 | Mean weeks (wks) of gestational age (GA) at the time of VER measurement, AER measurement and birth as well as birth weight in grams (g) of IUGR versus SGA versus AGA fetus.

Fetal group		GA at VER (wks)	GA at AER (wks)	GA at birth (wks)	Birth weight (g)
IUGR	Mean	33.6	33.8	35.5	1720
	N	14	11	15	15
	SD	2.7	2,6	2.8	405
SGA	Mean	34.7	34.1	38.6	2446
	N	22	23	32	32
	SD	3.0	3.4	2.1	401
AGA	Mean	33.9	33.9	40.0	3454
	N	37	34	60	60
	SD	3.0	3.1	1,6	412

already altered during gestation and might cause an alteration in the neurological developmental trajectory in later life. However, it must be emphasized that these findings are based on a relatively small group of children.

Intrauterine growth restriction, a pathologic growth restriction of fetuses, is associated with significant neonatal morbidity and mortality (Nardozza et al., 2017). It is also believed to impact morphological and structural brain development (D'Hooghe and Odendaal, 1991; Vindla et al., 1997; Nijhuis et al., 2000; Tolsa et al., 2004; Dubois et al., 2008; Lodygensky et al., 2008). We recently reported that latencies of fetal AER and VER assessed by fMEG are delayed in fetuses with IUGR (Morin et al., 2015). In the present study, however, the differences in VER and AER latencies between IUGR, SGA, and AGA fetuses were not statistically different. A possible explanation for these seemingly contradictory findings may be due to the fact that we had used a case control approach in the previous study to match subjects for GA and fetal behavioral state. Since our primary focus in the present study was on the effect of IUGR on neurodevelopmental changes at 2 years of age, we decided to increase sample size by including not only matched pairs of SGA-AGA and IUGR-AGA subjects but also of all other subjects. For proof of the possible predictive value of fMEG, further studies with larger population sizes and longitudinal assessment of functional brain development are necessary.

In the current study, we used simple tone stimulation only. However, since several cognitive capabilities such as discrimination and habituation are already established in the last trimester of gestation, it would be worthwhile to apply these stimulation paradigms to determine whether they are

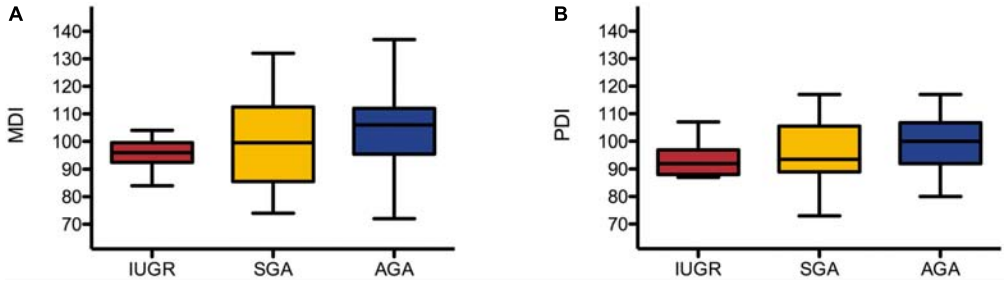

FIGURE 2 | Box plots of MDI **(A)** and PDI **(B)** in IUGR, SGA, and AGA group. Box plots represent median, interquartiles, and ranges.

TABLE 2 | MDI, PDI, VER latency and AER latency in IUGR, SGA, and AGA groups as calculated using one-way ANOVA.

		N	Mean	*SD*	*p*-value
MDI	IUGR	8	96	6	
	SGA	18	100	16	
	AGA	40	103	13	
	Total	66	101	13	0.044*
PDI	IUGR	7	94	7	
	SGA	16	96	11	
	AGA	40	100	10	
	Total	63	98	10	0.213
VER latency	IUGR	14	233	59	
	SGA	22	217	64	
	AGA	37	204	57	
	Total	73	213	60	0.282
AER latency	IUGR	11	204	65	
	SGA	22	220	61	
	AGA	33	188	69	
	Total	66	201	67	0.206

*Welch's t-test was used on account of unequal variances and sample-sizes and *indicates a significant group difference.*

TABLE 3 | Comparisons of the MDI between the IUGR, SGA, and AGA groups.

	IUGR	SGA	AGA
IUGR	–	−4.44 (± 4.33) *p* = 0.568	−7.83 (± 2.91) *p* = 0.035
SGA	4.44 (± 4.33) *p* = 0.568	–	−3.38 (± 4.26) *p* = 0.710
AGA	7.83 (± 2.91) *p* = 0.035	3.38 (± 4.26) *p* = 0.710	–

The differences of mean MDI values (± standard error) are shown and the respective p-values were calculated by Games-Howell post hoc analysis.

more specific for alterations of early fetal brain development (Draganova et al., 2005; Matuz et al., 2012; Muenssinger et al., 2013; Hartkopf et al., 2016). Interestingly, intrauterine auditory stimulation with the maternal voice in growth-restricted fetuses has been proposed as a potential tool to compensate brain alterations that might be responsible for later language impairment (Kisilevsky et al., 2014).

When it came to childhood development, we observed lower cognitive and psychomotor abilities in IUGR than in AGA children, although only the differences in cognitive (mental) scores were of statistical significance. In line with our results, earlier trials showed that former IUGR infants are more liable to achieve lower scores in neurocognitive and/or motor developmental assessment tests than control children without IUGR (for a review, see Murray et al., 2015). The comparability of studies on neurocognitive development of IUGR children, is, however, limited due to the selection criteria for growth restriction. Unlike reduced growth in SGA fetuses, which is usually constitutional, the growth delay in IUGR has a pathological cause. We therefore identified IUGR fetuses by using ultrasound to estimate fetal weight as well as to measure the umbilical artery pulsatility index. The latter is a marker of placental blood supply and a clinical standard to monitor intrauterine malnutrition (Murray et al., 2015). In a follow-up sample of 83 very-low-birth-weight infants, Leppanen et al. used the mental scale of BSID-II to show that only the subgroup with a pathological Doppler was affected by an altered cognitive outcome at the age of 2 years, whereas motor development remained unaffected (Leppanen et al., 2010). This is akin to the present study: PDI of BSID-II did not reveal any differences in psychomotor development between IUGR, SGA, and AGA children at 2 years of age. Several other studies investigating motor outcomes in IUGR children also reported that no differences were observed (Wienerroither et al., 2001; Eixarch et al., 2008; Padilla et al., 2010). Some study results indicate an influence of prematurity and severity of IUGR on motor development (Gazzolo et al., 1995; Padilla et al., 2011).

The MDI of the BSID-II includes measures for different cognitive skills, i.e., active and passive speech development, problem solving, or memory performance. The updated version "Bayley Scales of Infant and Toddler Development, Third Edition" (BSID-III) provides more specific subscores: a cognitive scale, a receptive language and an expressive language scale. To establish specific approaches to support affected infants and their families with early interventions, the assessment should be performed with the updated version in future investigations. However, the German version of the third edition was not available at the time of this study, nor is a behavioral scale, as provided by the original versions of BSID-II and BSID-III, available in the German language to date. Results of studies investigating behavioral

changes in former IUGR children indicate that attention, social-interactive skills or mood might also be affected (Roza et al., 2008; Beukers et al., 2017).

The major limitation of the current study is the low sample size, particularly for the IUGR group. Future studies with larger sample sizes should consider co-factors such as onset, duration and severity of IUGR to gain more detailed information about the impact of different types of IUGR (Miller et al., 2016). Moreover, loss to follow-up might be influenced by socioeconomic or demographic factors and might therefore bias our results (see **Figure 1** for drop-out at the different stages).

CONCLUSION

The results of this study support the hypothesis that IUGR might be a risk factor for a delay in neurocognitive development (MDI) in two-year old children. However, the differences were only modest, and not significant with respect to the PDI, and the three study groups did not differ significantly in fetal event-related brain activity. The investigation of underlying physiological processes and their impact on human brain development should be the focus of further research. Moreover, larger trials with a standardized definition of IUGR and well-defined outcome measures are required to identify factors that impact the role of IUGR on child development. These findings would be instrumental in developing specific treatment and support for the affected infants and their families.

AUTHOR CONTRIBUTIONS

JH, HP and IK-S conceived and designed the study. MW, JP-F, SB, and IK-S recruited the participants. JP-F, SB, and IK-S performed fetal ultrasound and Doppler measurements. JH, JK, and MW carried out fMEG measurements. CW conducted the physical examination of neonates. JK and JH performed Bayley testing. JH, AC, FS and HP analyzed the data and were responsible for statistics. JH, FS, HP and IK-S prepared the draft manuscript. All authors made substantial corrections for the final manuscript.

FUNDING

This work was partly supported by the German Research Foundation (DFG BI 195/50 and KI 1306/3-1), the University of Tuebingen (E.05.00303 and E.05.0259.1), Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.), and the Foundation of Baden-Wuerttemberg. We acknowledge support by the German Research Foundation and Open Access Publishing Fund of University of Tuebingen.

ACKNOWLEDGMENTS

We are very grateful to all the families who participated in our study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AM and handling Editor declared their shared affiliation at the time of the review.

frontiers in Endocrinology

REVIEW
published: 27 September 2018
doi: 10.3389/fendo.2018.00570

Regulation of Placental Development and Its Impact on Fetal Growth—New Insights From Mouse Models

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The placenta is the chief regulator of nutrient supply to the growing embryo during gestation. As such, adequate placental function is instrumental for developmental progression throughout intrauterine development. One of the most common complications during pregnancy is insufficient growth of the fetus, a problem termed intrauterine growth restriction (IUGR) that is most frequently rooted in a malfunctional placenta. Together with conventional gene targeting approaches, recent advances in screening mouse mutants for placental defects, combined with the ability to rapidly induce mutations *in vitro* and *in vivo* by CRISPR-Cas9 technology, has provided new insights into the contribution of the genome to normal placental development. Most importantly, these data have demonstrated that far more genes are required for normal placentation than previously appreciated. Here, we provide a summary of common types of placental defects in established mouse mutants, which will help us gain a better understanding of the genes impacting on human placentation. Based on a recent mouse mutant screen, we then provide examples on how these data can be mined to identify novel molecular hubs that may be critical for placental development. Given the close association between placental defects and abnormal cardiovascular and brain development, these functional nodes may also shed light onto the etiology of birth defects that co-occur with placental malformations. Taken together, recent insights into the regulation of mouse placental development have opened up new avenues for research that will promote the study of human pregnancy conditions, notably those based on defects in placentation that underlie the most common pregnancy pathologies such as IUGR and pre-eclampsia.

Keywords: placenta, trophoblast, mouse models, IUGR, fetal growth restriction, DMDD

OPEN ACCESS

Edited by:
Elke Winterhager,
Universität Duisburg-Essen, Germany

Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *27 July 2018*
Accepted: *06 September 2018*
Published: *27 September 2018*

Citation:
Woods L, Perez-Garcia V and Hemberger M (2018) Regulation of Placental Development and Its Impact on Fetal Growth—New Insights From Mouse Models. Front. Endocrinol. 9:570. doi: 10.3389/fendo.2018.00570

INTRODUCTION

Intrauterine growth restriction (IUGR; also referred to as fetal growth restriction, FGR) is a common pregnancy complication, affecting around 3–8% of pregnancies worldwide (1–3). IUGR overlaps but is distinct from the more general condition known as small for gestational age (SGA), which is most commonly defined as weight below the 10th percentile for the gestational age. Whilst SGA babies are small but may be physiologically normal, IUGR is a pathological condition defined as the failure of a fetus to attain its full genetic growth potential. IUGR is a leading cause of stillbirth, prematurity, cerebral palsy and perinatal mortality (4–6). In the most severe cases, IUGR can lead to

embryonic lethality and miscarriage during pregnancy. IUGR babies may have to be delivered prematurely, and therefore IUGR is also an indirect cause of preterm birth (7). Moreover, IUGR increases an infants' lifelong risk of adverse health outcomes including long-term poor neurological development, poor postnatal growth, and other childhood conditions, including serious and long-lasting immune deficiencies. Even well beyond infancy and childhood, IUGR is associated with a significant increase in the risk of cardiovascular disease, diabetes mellitus and hyperinsulinemia (8–10). Despite the distinction between IUGR and SGA, it is noteworthy that SGA infants are also affected by increased perinatal morbidity and mortality similar to IUGR, including neurodevelopmental disorders, stillbirth, and lifelong risk of adverse health outcomes (11).

IUGR is a complex and multifactorial disorder with a wide spectrum of potential causes. Some of these originate in specific genetic defects or congenital abnormalities in the embryo itself, or are a consequence of intrauterine infections. However, the majority of IUGR cases are caused by a failure of the placenta (10). The placenta is the extra-embryonic organ that only persists for the duration of pregnancy but that is absolutely essential for all intrauterine development. It has a number of essential functions such as anchoring the conceptus to the uterine wall, producing hormones to sustain pregnancy, inducing an immune-privileged environment and—as the perhaps most widely appreciated role—providing the embryo with sufficient amounts of nutrients and oxygen. Because of the pivotal role of the placenta in the etiology of IUGR, we here provide a brief overview of placental development, focussing for the most part on the mouse as the genetically most tractable model system to study early developmental processes at the molecular level. Specifically, we concentrate on fetal-specific effects of gene mutations. Interactions between placental trophoblast cells and maternal immune cells are also known to have an influence on growth trajectories of the fetus, but this aspect is outside the scope of this review and has been summarized elsewhere (12). Following this, we discuss novel insights into genetic causes of placental dysmorphologies. We will focus on recent findings that highlight the highly under-estimated number of genes contributing to placental development, and offer first approaches on how these may serve to identify novel molecular hubs that may be of key importance for placentation. These advances in the field harbor the prospect of enabling a better appreciation of the various causes of IUGR at the molecular and genetic level, which may lead to improved disease sub-classification, refined diagnosis and potentially to improved treatment options in the future.

BRIEF OVERVIEW OF PLACENTAL DEVELOPMENT

Tracing back in developmental time, the first definitive emergence of future placental cells occurs at the blastocyst stage with the formation of the trophectoderm. The trophectoderm forms the outer shell of the blastocyst, which is set aside from cells of the inner cell mass that will generate the embryo proper. Trophectoderm-derived cells ultimately give rise to all trophoblast cell types of the future placenta. Trophoblast cells make up the majority and defining aspects of the placenta. However, the most important exception to this placental cell provenance is the fetal placental vasculature. Endothelial cells of the fetal placental vasculature descend from cells of the extra-embryonic mesoderm that form the allantois and umbilical cord (13). These extra-embryonic mesodermal cells emerge slightly later in development at gastrulation, but ultimately have their cell lineage origin in the inner cell mass and epiblast. Thus, the placenta is a composite organ of two distinct cell lineages that arise from the fertilized embryo and that are established in early development, (1) the trophoblast lineage as the first lineage to differentiate that exclusively gives rise to placental trophoblast cell types, and (2) the extra-embryonic mesoderm that originates from cells of the inner cell mass and forms the fetal placental vasculature (**Figure 1A**).

Apart from these fetal-derived cell types, a vital aspect of placental development is the maternal uterine tissue into which the blastocyst is embedded upon implantation. The cells of the uterine lining, the so-called endometrium, undergo a specialized decidualization reaction upon implantation that is instrumental to support normal placentation and hence embryonic growth and survival (14). Indeed, the decidua will form part of the mature placenta. These principal tissue contributions are broadly similar between murine and human placentas. It is because of this complex and unique composition, combining fetal and maternal parts and thus amalgamating cells with different genetic constitution in a single organ, that often makes it difficult to discern where precisely the putative causes of a placental malformation may reside.

Placentation in the Mouse

Mouse models have been instrumental for advancing our knowledge of the impact of extra-embryonic development on pregnancy outcome and tracing the cell lineage origins of particular defects. For this reason, it is important to appreciate the progression of extra-embryonic development in the mouse, in comparison to that in humans. Following on from blastocyst formation, the trophectoderm cells surrounding the murine blastocoel cavity will soon cease to proliferate and enter endoreduplicative cell cycles, forming early (primary) trophoblast giant cells (TGCs). These TGCs help the embryo penetrate the uterine epithelium and implant into the endometrium, a process that takes place from developmental day (E) 4.5 onwards. Following implantation, polar trophectoderm cells overlying the inner cell mass continue to proliferate in response to a fibroblast growth factor 4 (FGF4) signal emanating from the epiblast (15). The arising column of diploid trophoblast cells forms the extra-embryonic ectoderm (ExE), a tightly packed epithelial tissue that retains stem cell potency (**Figure 1A**). Cells at the proximal tip of the ExE, farthest away from the epiblast, will further differentiate into the ectoplacental cone (EPC) that sits like a cap on top of the ExE. The cells at the margins of the EPC differentiate into secondary TGCs. It is these secondary TGCs in particular that acquire invasive characteristics, penetrating deeply into the endometrial stroma and making contact with

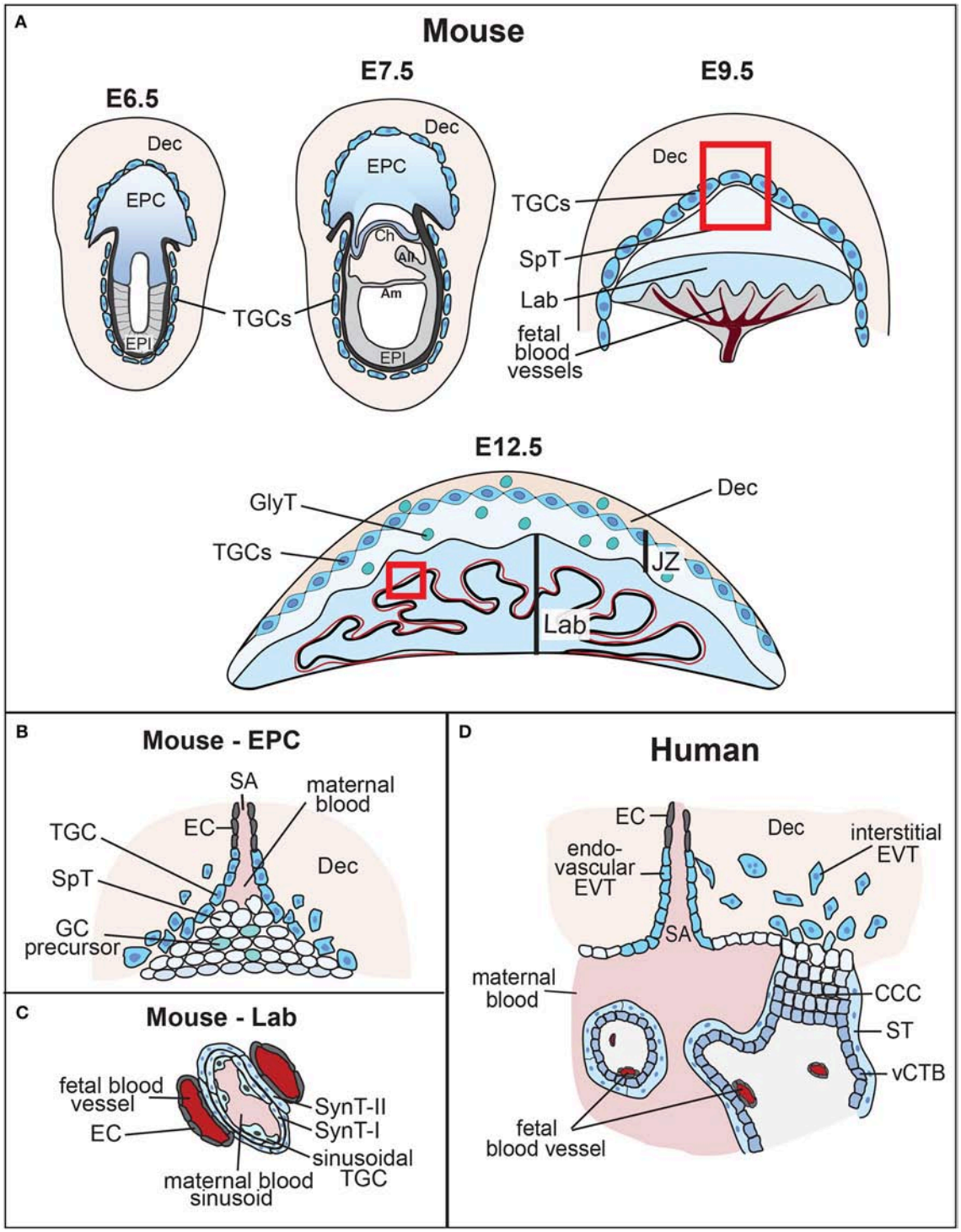

FIGURE 1 | Overview of mouse placental development and similarities to human placenta. **(A)** Following implantation, the blastocyst's mural trophectoderm differentiates into trophoblast giant cells (TGCs), while trophectodermal cells that overlie the inner cell mass form the extra-embryonic ectoderm (ExE) and the ectoplacental cone (EPC). The future embryo originates from the blastocyst's inner cell mass that differentiates into the epiblast (EPI). The conceptus is embedded into the maternal decidua (Dec) which differentiates from the uterine endometrium. With gastrulation, the chorion (Ch) consisting of a trophoblast and an extra-embryonic mesodermal cell layer, the amnion (Am) and the allantois (All) are formed. Cells at the margins of the of the EPC differentiate into invasive, secondary TGCs, that remodel the maternal vasculature [highlighted by the inset, see **(B)**]. The allantois grows out and attaches to the chorion (chorio-allantoic fusion) around E8.5, a critical process for developmental progression. At E9.5, allantoic blood vessels start to invaginate into the chorionic ectoderm to initiate formation of the placental labyrinth (Lab). Trophoblast cells overlying this layer differentiate into future spongiotrophoblast (SpT) and glycogen cells (GCs). The mature mouse placenta is established

(Continued)

FIGURE 1 | around mid-gestation (∼E10.5) and continues to grow in size and complexity. It consists of three main layers: the labyrinth (Lab), the junctional zone (JZ) made up of SpT, GCs and TGCs, and the maternal decidua (Dec). The labyrinth is the main site of nutrient and gas exchange [highlighted by the inset, see **(C)**]. Black bar indicates thickness of the labyrinth zone. **(B)** Magnified view of the tip of the EPC where invasive TGCs remodel maternal spiral arteries (SA) by eroding their smooth muscle lining and displacing their endothelial cell (EC) layer. **(C)** Close-up view of the interhaemal barrier, consisting (from maternal to fetal side) of a discontinuous layer of sinusoidal TGCs, two layers of syncytiotrophoblast (SynT-I and -II), and the endothelial cell layer of the fetal blood vessels. **(D)** Despite significant morphological differences, comparison with the human placenta reveals structural and/or functional similarities. Human placental villi are made up of a mesenchymal core that contains the fetal blood vessels, a layer of villous cytotrophoblast (vCTB) and one overlying layer of syncytiotrophoblast (ST) that is directly exposed to maternal blood. Thus, the haemochorial organization and the exchange barrier are similarly organized between mouse and human placentas. vCTBs are perhaps most analogous to the chorionic ectoderm in mice. In anchoring villi, cytotrophoblast cells form cytotrophoblast cell columns (CCCs) that invade into the maternal decidua (Dec). Cells at the base of the CCCs proliferate, pushing cells along the column where they progressively differentiate into invasive extravillous trophoblast (EVT). Trophoblast invasion occurs along two routes, interstitially into the decidual stroma, and along an endovascular route to replace the endothelial cell (EC) lining of maternal spiral arteries (SA). This spiral artery remodeling process is instrumental for healthy pregnancy progression, and is equally shared with the mouse where these processes occur between ∼E7.5 and E10.5 as shown.

maternal arteries. Trophoblast invasion by TGCs peaks between ∼E7.5 and E9.5. This process entails that TGCs erode away the smooth muscle layer and displace the endothelial cell lining of maternal blood vessels (**Figure 1B**). As a consequence, maternal blood is funneled into the placenta in trophoblast-lined conduits and in the absence of vaso-constrictive control by the mother (16). These vascular remodeling processes are key to the successful progression of pregnancy, as they lay the anatomical foundations for the functional capacity of the developing placenta.

Labyrinth Formation

With gastrulation, cells of the ExE differentiate into the chorionic ectoderm. At the same time, the allantois develops from extra-embryonic mesoderm at the posterior end of the embryo, positioned exactly at the embryonic—extra-embryonic boundary. The allantois grows out and toward the chorion, ultimately attaching to it at around E8.5. This process of chorio-allantoic fusion is absolutely essential for further pregnancy progression. In its absence, development is abrogated soon afterwards. Chorio-allantoic fusion establishes a scenario where extra-embryonic mesodermal cells are in direct contact with the chorionic ectoderm, and start to invaginate into it in finger-like projections at sites pre-determined by expression of a particular transcription factor, *Gcm1* (17, 18). In the chorionic trophoblast, these invaginating mesodermal protrusions trigger a differentiation process in which individual trophoblast cells fuse to form syncytiotrophoblast. Syncytiotrophoblast cells ultimately will establish the transport surface, or "interhaemal membrane," of the placenta (**Figure 1C**). They form the blood sinusoids through which maternal blood (brought in by the trophoblast-lined spiral arteries and canals) percolates, and across which nutrients and oxygen must be transported to reach the fetal blood circulation. In the mouse, the entire exchange barrier, from the maternal to the fetal side, is made up of a total of three continuous cell layers, two layers of syncytiotrophoblast (SynT-I and SynT-II, respectively) and the extra-embryonic mesoderm-derived fetal endothelial cells (19). Sinusoidal TGCs that are likely of chorionic trophoblast origin are also present at the maternal side, apposed to the SynT-I layer, but they only form a fenestrated, discontinuous layer that does not constitute a complete barrier (**Figure 1C**). These intricate developmental steps start to occur from around mid-gestation in the mouse (E9.5–10.5) and lead to the formation of the so-called labyrinth. With labyrinth formation, the mature mouse placenta is being established. The labyrinth continues to grow for the next days by continued branching morphogenesis leading to further elongation and refinement of these inter-digitated vascular spaces. This architecture achieves a large surface area for transport in which maternal and fetal blood circulations come into close contact but never mix. Moreover, maternal and fetal blood flow in a counter-current direction, thus optimizing transport capacity (20).

As can be appreciated from these complicated and intricate developmental processes, defects and deficiencies in labyrinth formation are a frequent cause of developmental failure and growth deficits, respectively. Up until mid-gestation, the yolk sac meets the nutritional needs of the early embryo. However, from around E10 onwards the transport capacity of the placenta is an absolute requirement to ensure embryo survival. Indeed, this requirement to switch from yolk sac nutrition to placental nutrient supply, tied to the necessity for chorio-allantoic fusion and labyrinth formation to occur successfully, creates a developmental bottleneck around mid-gestation in the mouse when a large proportion of mutants die.

Junctional Zone Formation

The junctional zone (JZ) is positioned between the labyrinth and the maternal decidua. Together with the labyrinth, it forms the other major layer of the fetal part of the mature mouse placenta. The JZ originates mainly from cells of the core of the EPC, as judged by gene expression of prominent markers, such as *Tpbpa*. It contains three main cell types: spongiotrophoblast cells (SpT), glycogen cells (GCs) and a layer of TGCs that directly border the decidua (21). GCs often associate with maternal blood canals and sinuses. From about E12.5 onwards, they invade into the decidua where they become associated with maternal blood spaces. Because of their glycogen content and location, GCs are believed to serve as an energy store that can provide additional nutrition to the placenta and/or embryo. Apart from that, the JZ constitutes the main endocrine compartment of the placenta. It produces vast amounts of hormones, growth factors and cytokines that are important for the normal progression of pregnancy, acting on both the maternal and fetal physiology (22, 23).

The Human Placenta

Although mammalian placentas are functionally convergent, placental morphology is remarkably different between species. Mouse and human placentas share in common a haemochorial type of placentation, meaning that fetal trophoblast cells are directly bathed in maternal blood. In both species trophoblast is invasive and penetrates deeply into the endometrium, in humans even farther reaching into the muscular layer of the myometrium. Cellular morphology and overlapping gene expression patterns have helped identify analogous cell types in mouse and human placentas, although a direct comparison is not always clear-cut. The structure analogous to the murine labyrinth are the placental villi in the human placenta. As in the mouse, the cells exposed to the maternal blood are syncytial in nature, however, in humans there is only one layer of syncytiotrophoblast (**Figure 1D**). Immediately underlying the syncytiotrophoblast is a layer of villous cytotrophoblast cells (vCTBs) that continuously fuse into the syncytium and replenish it. These cells may be analogous to chorionic ectoderm cells in the early mouse placenta, but whether such a cell population persists into later murine gestation remains unknown. The core of the villus is made up of mesenchymal cells, fetal blood vessels, and a macrophage cell type known as Hofbauer cells. The blood vessels come close to the vCTB layer at presumptive sites of nutrient transfer, which hence in humans has to cross at least one syncytiotrophoblast layer, one cytotrophoblast layer and the fetal endothelial cells (19).

While there is appreciable morphological and functional similarity between the mouse labyrinth and the human placental villous structure, the equivalent to the JZ is harder to make out. Conceivably, such a relationship exists with the cytotrophoblast cell columns (CCCs). These columns grow out at certain points from the villi and anchor the placenta to the uterine wall. They consist of tightly packed cytotrophoblast cells that are highly proliferative at the base of the CCC, thereby contributing to the growth of the column. At their distal tips, extravillous cytotrophoblast (EVT) cells leave the context of the column and invade deeply into the uterine stroma. As in the mouse, this process of trophoblast invasion is instrumental to remodel maternal spiral arteries into large, trophoblast-lined canals that bring blood into the placenta (24). From the base of the column to the tip, cytotrophoblast cells undergo a well-documented epithelial-mesenchymal transition that coincides with acquiring invasive characteristics (25, 26). Like TGCs, EVT cells are also becoming polyploid and/or aneuploid, but not to the same extent as in the mouse where the genome content of TGCs can gain an equivalent of up to 1000N. In many regards, this morphological layout is somewhat similar to the ExE-EPC structure in the early mouse conceptus. It translates less obviously into the JZ structure of the mature mouse placenta, although by cellular descent these cells will be related to their earlier ExE/EPC progenitors.

PLACENTAL STRUCTURES LINKED TO EMBRYONIC GROWTH

Despite this mixed picture of structural and functional similarities and discrepancies between mouse and human placentas, the mouse model has been instrumental for gaining insights into molecular pathways that direct early trophoblast cell fate decisions as well as for identifying genes that affect placental development. For this reason, we focus below on key examples of what we have learnt from mouse mutants over recent years.

Maternal Spiral Arteries and Blood Canals

Trophoblast invasion and the remodeling of maternal spiral arteries are perhaps the most widely accepted processes associated with the patho-etiology of IUGR. A large number of genes have been linked to conferring invasive characteristics to mouse and human trophoblast, thereby endowing this cell type with the capacity to target and remodel maternal spiral arteries deep within the maternal decidua. Disruption of this process impairs placental blood flow and is a major cause of IUGR and pre-eclampsia (27). Pre-eclampsia is a pregnancy complication characterized by high blood pressure, proteinuria, and often fetal growth restriction. Both disorders share common risk factors and outcomes, however they are distinct conditions, i.e. not all cases of IUGR are also complicated by pre-eclampsia, and *vice versa*. In the mouse, invasive TGCs originate from the margins of the EPC. Their vital role in remodeling the maternal vasculature has been demonstrated in cell type-specific ablation experiments that took advantage of the regulatory elements of the *Tpbpa* gene to drive Cre recombinase expression. *Tpbpa* is a key marker gene of the precursors of invasive TGCs located within the core of the EPC. Ablation of *Tpbpa*-positive cells by conditional activation of a *Diphteria* toxin gene results in trophoblast invasion deficiencies and consequently in defective remodeling of maternal spiral arteries (28). Mature placentas from such mice exhibit a small JZ with reduced SpT, GC and TGC numbers, and the conceptuses die around E11.5. A comparable placental phenotype is observed upon deletion of the serine peptidase *Htra1*, which targets a similar cell population and partially ablates *Tpbpa*-positive EPC cells (29). These data show that in mouse, as in humans, trophoblast invasion and the vascular remodeling process mediated by these invading cells are pivotal determinants of fetal growth and survival.

In addition to these proof-of-concept phenotypes in the mouse, strong evidence suggests that the diameter of maternal blood canals is regulated by the NOTCH signaling pathway. Deletion of the transmembrane receptor *Notch2* in trophoblast causes developmental delay due to impaired invasion of maternal spiral arteries and a reduced size of maternal blood canals and -sinuses at the entry point into the placenta (30, 31). The importance of NOTCH signaling has also been demonstrated in the human placenta. In the first trimester placenta, NOTCH1 is expressed exclusively by progenitors of invasive EVTs (32), suggesting a role in spiral artery remodeling. In term placentas the NOTCH1,−2,−4 receptors, and the NOTCH pathway ligand JAGGED2 are expressed in the brush border of the syncytiotrophoblast layer, with NOTCH1 and NOTCH4 also marking vascular endothelial cells. Expression of these NOTCH family components is disrupted in placentas affected by IUGR and pregnancy-induced hypertension (33).

Junctional Zone Defects

Numerous mouse models with JZ defects exhibit an IUGR phenotype (**Figure 2**, **Supplementary Table 1**). A small JZ is associated with impaired production of the large family of prolactin-like hormones. However, deletion of the gene encoding prolactin (*Prl*) alone does not cause overt JZ defects (34), potentially implying functional redundancy with the other 22 prolactin-like genes present in the mouse genome (35). Alternatively, the abnormal expression of prolactin family hormones that is observed in many mutants with JZ defects may represent a readout rather than a cause of abnormal JZ size. In any case, fetal growth is affected by the size of the developing JZ. Apparently, this effect is indeed linked to the JZ and not the placenta as a whole, as fetal growth is impaired in mouse models which exhibit differences in the JZ but not the labyrinth (36, 37). JZ defects cause an altered endocrine environment in the placenta locally, but also have systemic effects on both fetus and mother. Such dysmorphologies may therefore affect fetal growth due to deregulation of the control of placental growth and structure, or by controlling the allocation of maternal resources, for example by altering maternal insulin resistance (38).

A major component of the JZ are GCs. Their ability to store and release large amounts of glycogen suggests they play a role in supplying glycogen as an energy source to the growing conceptus. For example, the volume of the JZ, the proportion of GCs it contains, and their total glycogen content is reduced when pregnant females are subjected to chronic or acute dietary restriction (39–41). Imprinted genes, i.e. the small collection of genes that are expressed in a parent-of-origin dependent manner, are a chief regulator of JZ development and maternal resource allocation. Disruption of imprinted gene expression in the placenta can be associated with both, fetal growth restriction or overgrowth in mice and humans. Prominent examples in humans are Beckwith-Wiedemann syndrome which is associated with fetal overgrowth, and Silver-Russell syndrome characterized by growth restriction. Both syndromes are caused by imprinting errors on chromosome 11p15, notably a paternal and maternal duplication of the imprinting control region, respectively. In the mouse, imprinting defects are commonly associated with JZ defects affecting the size of this compartment and hence the number of SpT and GCs (42). For instance, increasing the gene dosage of the imprinted gene *Phlda2* (encoding a pleckstrin homology domain protein) by a single copy dramatically reduces the size of the JZ and the amount of stored glycogen by between 25 and 35% (43). The remaining GCs fail to migrate into the decidua in late

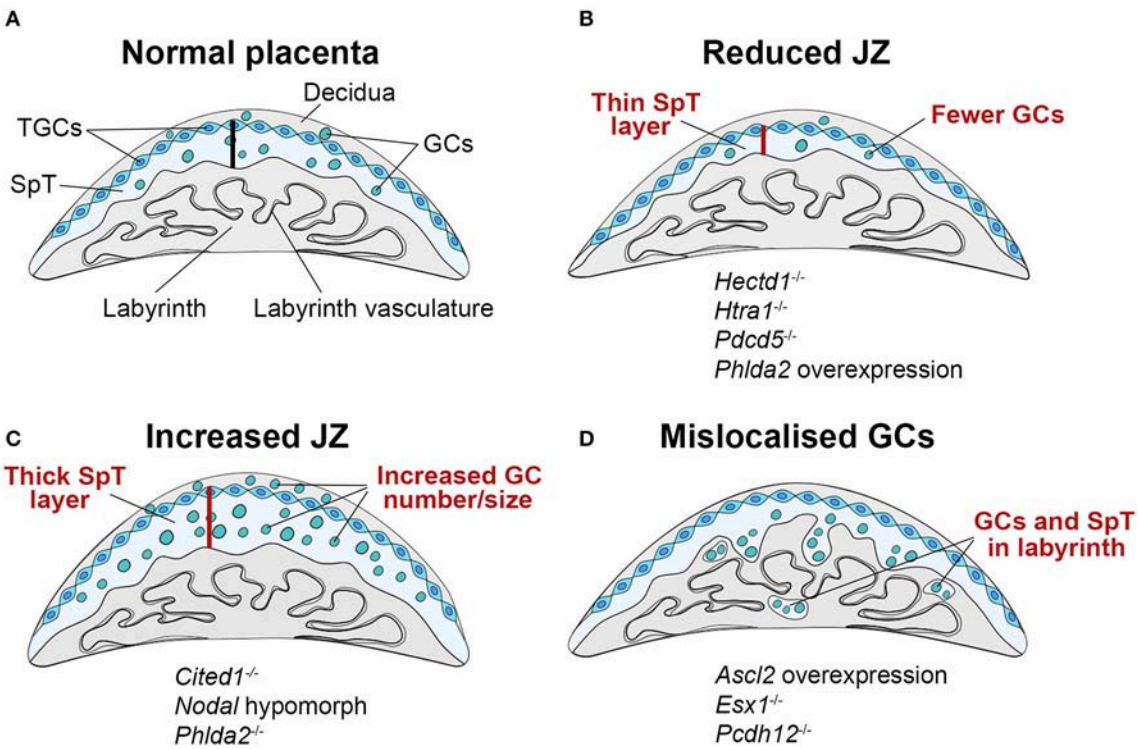

FIGURE 2 | Schematic representation of JZ defects that can be associated with IUGR. **(A)** The junctional zone (JZ) is composed of three cell types: spongiotrophoblast cells (SpT), glycogen cells (GCs) and trophoblast giant cells (TGCs). The JZ provides energetic (glycogen), hormonal and physical support to ensure correct placentation and pregnancy progression. **(B–D)** Recurring JZ phenotypes entail **(B)** a reduced thickness of the JZ layer (indicated by the red bar) with fewer SpT and/or GCs, **(C)** an increased size of the JZ with more SpT and/or GCs and **(D)** a mislocalisation of GCs and SpT in the labyrinth. All of these phenotypes can be associated with IUGR, highlighting that JZ size alone is not indicative of placental efficiency, but that other parameters such as effect on cellular function, and precise cell localisation in relation to blood vessels and blood conduits, is important. Examples of mouse mutants or overexpression models in which these dysmorphologies are observed are given (fetal genotype is shown).

gestation but persist in the JZ. Instead, *Tpbpa*-positive SpT cells and GCs become progressively mislocalized in the labyrinth compartment. Partial loss-of-function of another imprinted gene, *Ascl2,* which regulates *Phlda2* expression, similarly leads to depletion of SpT and ablation of GCs, and fetal growth restriction (44).

The common co-occurrence of a growth-retarded fetus with reduced GC numbers and less total glycogen content suggests that IUGR could be linked to the scarcity of glycogen in these placentas (**Figure 2B**). However, overabundance of glycogen is also associated with reduced fetal growth (**Figures 2C,D**). This is observed in the *Phlda2* knockout, which is associated with expansion of the junctional zone resulting in an over-accumulation of placental glycogen to 3x its normal levels, and leads to fetal growth restriction (45). Similarly, overexpression of *Ascl2* or knockout of *H19*, a maternally expressed non-coding RNA, leads to increased placental glycogen stores and reduced fetal growth (46, 47). An increase in GC numbers and glycogen content can also be associated with GC mislocalisation. This is the case in the *Ascl2* overexpression model or also in knockouts of the cell adhesion molecule *Pcdh12* or the homeobox gene *Esx1*, where GCs are ectopically located in the labyrinth (47–49). Hence, GC mislocalisation is emerging as a common feature of several gene manipulations that cause JZ defects associated with fetal growth restriction (**Figure 2D**). Although it may appear counter-intuitive that increased placental glycogen stores restrict fetal and placental growth, it may be that in such cases GCs are unable to supply their stored glycogen to the conceptus. This could be due to the inability of GCs to migrate into the decidua and access the maternal blood supply, or failure to break down and release stored glycogen, resulting in its over-accumulation. These deficits in turn may be caused by cell intrinsic defects in glycogen release, or altered hormonal control of glycogen metabolism, potentially due to defects in hormone production by the JZ. Taken together, these findings suggest that accumulation and storage of glycogen, and its subsequent release, are distinct and delicately balanced processes. Failure of GCs to either store adequate quantities of glycogen or to degrade and supply it as an energy source is detrimental to fetal and placental growth and development.

Although the human placenta does not contain specialized GCs akin to those found in the mouse, EVT cells located distally in the basal plate have the ability to store and metabolize large quantities of glycogen (19). The human placenta accumulates glycogen mainly in the first trimester, which then declines toward term, potentially contributing to the increased fetal and/or placental growth at this time (50, 51). Several studies have investigated glycogen content and metabolism in placentas complicated by IUGR, however as of yet no differences have been found in glycogen deposition (52, 53), or the expression and activity of enzymes involved in glycogen synthesis (54). The role of placental glycogen in normal human pregnancy and in pregnancy disorders is relatively understudied, however a recent review highlighted the association between altered glycogen deposition and metabolism in pathological pregnancy, including pre-eclampsia with IUGR, and diabetes (55). The strong association between altered placental glycogen stores and IUGR in the mouse prompts further research into understanding the role of placental glycogen in healthy and pathological pregnancies.

Labyrinth Defects

The role of the labyrinth as the site of nutrient exchange strongly implicates this placental layer in the pathogenesis of IUGR. Any defect in the labyrinth which alters its ability to mediate the transfer of nutrients and oxygen to the fetus may impact on fetal growth. Such defects lead to an imbalance between the metabolic demands of the fetus and the ability of the placenta to meet that need. Indeed, in a recent systematic screen of embryonic lethal or sub-viable mouse mutants that are frequently associated with IUGR prior to death, labyrinth defects stood out as a prominent site of placental failure (56). Many other reports of individual gene knockouts support the direct link between placental labyrinth development and fetal growth and viability (**Figure 3**, **Supplementary Table 1**) (57).

Small Labyrinth Size

The size of the labyrinth compartment is an important determinant of its capacity to nourish the fetus. A small labyrinth has a reduced transport surface area, thereby limiting the nutrients available to the fetal circulation and hence restricting the extent of fetal growth (**Figure 3B**). A small labyrinth can be the result of a labyrinth-restricted failure to expand this particular compartment, or because of an overall reduced placental size. For example, deletion of the translation initiation factor *Eif2s1* causes endoplasmic reticulum stress in the placenta and specifically reduces the size of the labyrinth layer, resulting in growth restriction of the fetus (2). These scenarios can sometimes be difficult to distinguish, as the labyrinth forms a substantial fraction of the placenta, and hence labyrinth-to-JZ ratios are often used as a measure of disruptions in the relative proportion of labyrinth size. Labyrinth growth is regulated by genes encoding key growth factor pathway components, including the receptors for fibroblast growth factor (*Fgfr2*), leukemia inhibitory factor (*Lifr*), epidermal growth factor (*Egfr*) and hepatocyte growth factor (*Met*) (58). *Met* (also known as *c-Met*), for example, is involved in the proliferation of labyrinth trophoblast progenitors, and its deletion leads to reduced labyrinth size and cellularity, and poor development of the vascular branching structure (59). The *Fgfr2-IgIII*, and *Egfr* mutants are embryonic lethal at midgestation due to disrupted labyrinth and JZ development (60–63), whilst the *Lifr* mutant displays perinatal lethality with poor organization of the labyrinth and spongiotrophoblast (64).

The insulin-like growth factor (IGF) axis is another important regulator of placental growth and development, and the placenta itself is a major source of IGF2 during pregnancy (65). Deletion of the imprinted, placenta-specific *Igf2 P0* transcript significantly reduces labyrinth thickness and surface area (66, 67), causing fetal growth restriction. Human IUGR pregnancies are characterized by altered IGF signaling, with low levels of IGF1 in umbilical cord blood and increased IGFBP1 and IGFBP2 (68–70). Human *IGF1* mutations are associated with severe IUGR (71–73), suggesting the conserved importance of the IGF

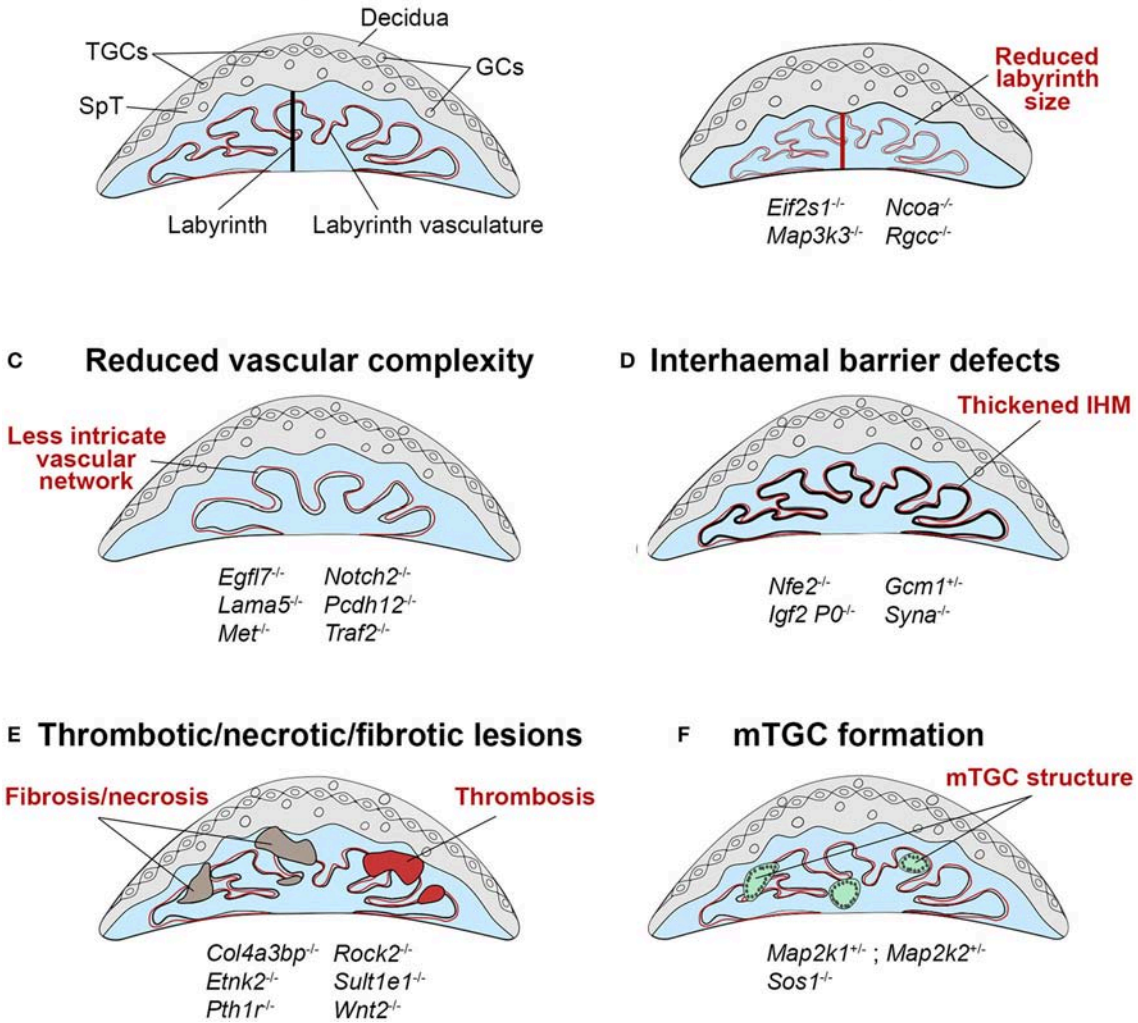

FIGURE 3 | Schematic representation of labyrinth defects associated with IUGR. **(A)** Within a normal placenta, the labyrinth is the largest layer and is the site of all nutrient and gas exchange between the maternal and fetal blood circulations (black bar indicates thickness of the labyrinth layer). As such, failure in the establishment of this intricately organized layer is a direct cause of IUGR, or in more severe cases of intrauterine lethality. Defects in the development of the labyrinth often originate from defective or insufficient invagination of allantoic blood vessels into the chorionic ectoderm (see **Figure 1**, E9.5) and the subsequent branching morphogenesis that has to occur to form this exchange surface. **(B)** Such defects can lead to a small labyrinth layer (indicated by the red bar, often coupled with an overall reduced size of the placenta) or **(C)** a reduced complexity of the vascular organization within the labyrinth, in which there are fewer, disorganized and/or inappropriately dilated fetal blood vessels and maternal conduits. Principally, these structural defects lead to a reduction of the surface area available for transport, which hence cause placental insufficiency. **(D)** Impaired nutrient transfer may also occur because of a thickened or dysfunctional interhaemal barrier (IHM). **(E)** The transport surface may be diminished due to overt lesions in the labyrinth layer, that frequently entail thrombotic or necrotic patches or the accumulation of fibrotic tissue. **(F)** An unusual trophoblast differentiation defect is observed in some mouse mutants with the formation of multinucleate trophoblast giant cells (mTGCs). These mTGCs likely have their origin in inappropriate fusion and syncytialisation of labyrinth trophoblast cells, disrupting the intricate labyrinthine architecture. Examples of mouse mutants or overexpression models in which these dysmorphologies are observed are given (fetal genotype is shown).

axis in placental development and fetal growth in mice and humans. The signaling cascades activated by the aforementioned growth factors and their receptors ultimately converge on the MAPK signaling cascade. Therefore, unsurprisingly, mutations in several MAPK pathway components also lead to varying degrees of labyrinth expansion deficits. As such, mutants of SOS1, GAB1, GRB2, RAF1, MEK1 (*Map2k1*), MEKK3 (*Map3k3*), and p38a (*Mapk14*) are characterized by a small labyrinth layer (**Supplementary Table 1**) (57, 74).

A small placental labyrinth is also a common feature of maternal undernutrition and diabetic mouse models leading to IUGR, showing that maternal exposure to external factors can affect labyrinth proliferation and expansion (39, 40, 75).

Vascular Organization

The intricately branched network of fetal blood vessels in the labyrinth develops initially by vasculogenesis, when angioblasts that are specified in the allantois extend into the chorion

to form the fetal labyrinth vasculature. As outlined above, failure in chorio-allantoic fusion prevents any such vascular development and is associated with mid-gestational lethality in the mouse. A key factor required for the specification of invagination points into the chorionic ectoderm is the transcription factor *Gcm1*. Homozygous *Gcm1* mutants develop normally until E9.5 but are growth restricted and die at E10.5 due to failing branching morphogenesis of the labyrinth. Instead, the chorion remains a flat layer in these mutants (17). Apart from complete failure of vessel invagination, milder forms of chorion folding defects and insufficient vessel invagination can also be observed in many other mutants (**Figure 3C**, **Supplementary Table 1**). Development of the chorio-allantoic vasculature is in part regulated by the TGF-β pathway, and *Tgfb*, *Alk1*, and *Alk5* null mutants all show labyrinth vascularisation defects (76, 77). Additionally, mutations in the WNT and BMP family components *Wnt2*, *Rspo3*, *Bmp4* and *Smad1* display abnormalities such as a small or absent allantois and failure of chorio-allantoic fusion, as well as defects in formation of the labyrinth vasculature, including failure of fetal vessels to invaginate into the chorion, and the inappropriate formation of large edematous maternal blood spaces (77). The PPAR signaling pathway also plays a role, as *Pparg* null trophoblast cells fail to undrego terminal syncytium formation, which disrupts the integrity of the vascular exchange interface and restricts growth of the fetus (78, 79). Members of the PPAR family, as well as *GCM1* and its antagonist *NFE2* have been implicated in human IUGR placentas (80–82), demonstrating that abnormal differentiation of the villous trophoblast, especially of the syncytiotrophoblast layer, also contributes to placental insufficiency and IUGR in humans.

From mid-gestation onwards, the fetal vessels undergo extensive branching by angiogenesis, which contributes to placental expansion to meet the increasing needs of the growing fetus. These processes are regulated by members of the vascular endothelial growth factor (VEGF), placental growth factor (PGF), FGF, WNT, and the TGF-β and BMP families (83). Disruption of such key angiogenic pathways in the placenta has significant impact on placental vascularisation and is causally associated with IUGR in mouse and human pregnancies (84). Commonly, placentas exhibiting such vascularisation defects show a reduction in the number of fetal and/or maternal blood spaces, or an abnormal lumen diameter (85–88). For example, mouse mutants with deletion of *Rgcc*, a VEGF-inducible angiogenic inhibitor (89), develop a small placenta with fewer and wider fetal capillaries. Poorly vascularised *Rgcc*$^{-/-}$ placentas show decreased expression of *Vegf2* and *Pgf*, and embryos are growth restricted from E16.5 onwards (87).

The VEGF and PGF families constitute some of the prime regulators of angiogenesis in the labyrinth. Deletion of *Vegf* or overexpression of *Pgf* leads to severe labyrinth vascularisation defects, causing IUGR and embryonic death due to placental insufficiency (90–92). Similarly, ablation of the VEGF receptor *Vegfr1* (*Flt1*) in the mouse causes vasculogenesis and angiogenesis problems, resulting embryonic lethality (93). Overexpression of the soluble isoform of the human VEGF receptor, sFLT1, in mouse embryos induces pre-eclampsia like symptoms in pregnant dams, including IUGR, hypertension, and proteinuria, which can be ameliorated by PGF induction suggesting that PGF works antagonistically with sFLT1 to regulate placental angiogenesis (94). Dysregulated expression of sFLT1, PGF, and other angiogenic factors has been observed in human pregnancies complicated by IUGR and early onset pre-eclampsia complicated by IUGR (95–97). Indeed, determination of the sFLT1:PGF ratio, in combination with ultrasound findings, shows promise as a test of pre-eclampsia risk in the clinic, with an elevated ratio as an indicator of disease risk in pregnant women before the clinical onset of symptoms (98, 99).

Interhemal Barrier Defects

In addition to reduced labyrinth size, placental transfer insufficiency can also be caused by defects in the exchange barrier itself (**Figure 3D**). This may entail a mis-regulated expression of particular transporter molecules, or an increased thickness of the interhaemal membrane (IHM), thus hampering transport efficiency. These defects may occur in isolation or as compounding factor of small labyrinth size or reduced vascular complexity.

The IHM is a selectively permeable trilaminar barrier whose surface area increases throughout gestation as the labyrinth expands and becomes more intricately branched. The IHM also becomes significantly thinner between E12.5 and E16.5 of pregnancy, thereby reducing the distance over which substances must diffuse between the maternal and fetal blood supplies. This increases the theoretical diffusion capacity of the placenta, and in conjunction with expansion and branching of the labyrinth maximizes materno-fetal exchange (100, 101). Therefore, an increased thickness of the IHM can impede the efficiency of nutrient transfer to the fetus (**Figure 3D**). Disrupted morphology and thickness of the IHM is evident in the *Igf2 P0* knockout and in Syncytin A (*Syna*)-ablated mice, both of which display fetal growth impairment followed by embryonic lethality (67, 102). Manipulation of *Gcm1* expression to levels both below or above normal, by heterozygous *Gcm1* deletion or upregulation due to deletion of its antagonist p45NF-E2 (*Nfe2*), respectively, increases the thickness of the IHM (86, 103). Although fetal growth in *Gcm1*$^{+/-}$ conceptuses is not affected, wild-type females carrying *Gcm1*$^{+/-}$ conceptuses develop late gestational hypertension similar to what is seen in pre-eclampsia patients. Human placentas from IUGR pregnancies express reduced levels of *NFE2*, leading to upregulation of *GCM1* and excessive development of the syncytium (82), suggesting that similar molecular pathways operate in the establishment of the human placental IHM and may contribute to IUGR when disrupted.

In addition to IHM thickness, nutrient supply deficits may also be caused by mis-expression of specific transporter proteins. The *Igf2 P0* knockout affects in particular the system A amino acid transport system. Embryos overexpressing human *sFlt1*, which as mentioned earlier is implicated in early-onset pre-eclampsia with IUGR, develop a small labyrinth with reduced expression of the glucose diffusion channel *Connexin26* (*Cx26*). In contrast to their decreased expression of *Cx26*, *sFlt1* overexpressing embryos increase fatty acid and cholesterol transport by upregulation of *Cd36* and *Abca1* expression, respectively (104). Similarly, mice

subjected to dietary restriction during pregnancy increase system A amino acid transporter activity leading to increased fetal amino acid accumulation (39). Amino acid and fatty acid/cholesterol uptake is increased in placentas with reduced ability to transport glucose, which may represent an attempt to compensate for an insufficient glucose supply. In fact, one study found that in normal pregnancies, the smallest placentas in a litter are more efficient in their nutrient transfer capacity to promote adequate fetal growth (105). Thus, up to a certain extent, small placentas may be able to compensate for reduced labyrinth size by increasing transport efficiency, but as labyrinth defects become more severe such compensatory mechanisms are insufficient to support normal embryonic growth.

Vascular Lesions

Additionally, defects in the integrity of the labyrinth can lead to the formation of edematous regions and thrombosis, generating infarcts which may impair fetal growth by disrupting the flow of fetal and maternal blood (**Figure 3E**). Mouse strains and mutants affected by placental thrombosis and infarction often show reduced fetal growth (106–109). Similar defects are a common feature of placentas from human pregnancies complicated by IUGR (110, 111). Likewise, failing development and impaired perfusion of the labyrinth can lead to tissue fibrosis and necrosis, thus limiting the available healthy labyrinth tissue for nutrient and gas exchange (**Figure 3E**) (112–114). For example, *Traf2*$^{-/-}$ (a regulator of NF-kappa-B and JNK) and *Col4a3bp*$^{-/-}$ (a collagen binding protein) placentas exhibit regions of fibrosis and necrosis within the vascularised labyrinth compartment, and deletion of the parathyroid hormone receptor *Pth1r* causes disruption of the labyrinth vasculature due to the presence of spongiotrophoblast inclusions and abnormal accumulation of maternal blood in the labyrinth (56).

Additionally, several mouse mutants with deletion of MAPK pathway components, including *Map2k1*, *Map2k2*, and the Ras guanine nucleotide exchange factor *Sos1*, display an unusual defect characterized by the formation of large multinucleate structures in the labyrinth (**Figure 3F**) (115, 116). These are referred to as multinucleate TGCs (mTGCs) in the literature but are unlikely to be true TGCs and may instead be a result of aberrant fusion of SynT-I and SynT-II, as they display characteristics of both layers (116). Mutants exhibiting mTGC formation also show reduced embryo growth (117) suggesting their presence may impede placental nutrient transfer due to severe disruption of the labyrinth branching structure and interruption of the vascular network.

As already alluded to, the placental defects described do not necessarily occur in isolation, as labyrinth growth, villous branching, and angiogenesis are interlinked. For example, a labyrinth with a simple branching structure is likely also small, with a reduced network of fetal capillaries. A placenta with failure in SynT differentiation or placental vascularisation may be both small, and have a thickened IHM due to defects in formation of the syncytium or fetal capillaries (67, 86, 102, 103). Defects in labyrinth morphogenesis and integrity are likely to also be affected by the presence of vascular thrombosis and infarction (106, 107).

Moreover, defects in labyrinth formation often co-occur with abnormal development of the JZ, suggesting that development of the two layers is interlinked. For example, deletion of *Ascl2* reduces the size of the JZ layer with an absence of GCs, in addition to severe defects in labyrinth morphogenesis and vascularisation (44). Maternal dietary restriction during pregnancy initially reduces the volume and glycogen content of the JZ with no effect on labyrinth size at E16, however by E19 the labyrinth compartment is significantly reduced in these placentas (39). These observations raise the possibility that hormone secretion by the JZ influences labyrinth morphogenesis, and JZ glycogen stores may be utilized during late gestation to fuel expansion of the labyrinth. In fact, the glycogen content of the JZ decreases in late gestation as GCs are lysed, coinciding with expansion of the labyrinth compartment at this time (100, 118). If so, this may contribute to abnormal development of the labyrinth in placentas with JZ defects (119, 120).

ENDOMETRIAL CAUSES OF IUGR

Development of the placenta is dependent upon successful implantation of the blastocyst into the uterine wall, followed by decidualization of the surrounding uterine stroma. This is necessary to facilitate a controlled trophoblast invasion into and remodeling of the stromal compartment, key pillars for subsequent placental development. Implantation and decidualization are regulated by a series of highly orchestrated and synchronized interactions between a competent blastocyst and the receptive endometrium. In the mouse, implantation occurs on the evening of day 4 of pregnancy when the blastocyst becomes attached to the receptive endometrium and starts to embed itself into the uterine wall. Implantation of the embryo is a prerequisite for pregnancy progression without which development cannot occur (121–123). Disruption of implantation and decidualisation, or a delay in its timing can create a ripple effect through pregnancy, leading to poor placentation and abnormal growth and development of the embryo (124). Maternal deletion of the cytosolic phospholipase A2 (*Pla2g4a*), prostaglandin synthase 2 (*Ptgs2*), or lysophosphatidic acid receptor 3 (*Lpar3*), factors involved in the prostaglandin synthesis pathway, or of the homeobox gene *Msx1*, causes implantation to be deferred beyond the normal window (125–127). This leads to a spectrum of complications ranging from mild to severe growth restriction through to embryonic demise, which may be secondary to developmental abnormalities of the placenta. A similar outcome was observed when blastocysts developed to E4 were allowed to implant in an E5 uterus (125). This pathological deferred implantation is distinct from embryonic diapause when a blastocyst is retained dormant in the uterus for an extended period before resuming implantation (128). Thus, defects in implantation can have a knock-on effect on development of the placenta, with ramifications for fetal growth and development. Human studies suggest that deferred implantation may be a factor in pregnancies affected by growth restriction. Late implantation occurring >12 days after ovulation in human pregnancy is associated with early

pregnancy loss (129). Additionally, pregnancies with a longer interval between ovulation and implantation have a smaller first trimester crown-rump length than those that implant earlier (130), which has been shown to be a predictor of low birth weight and IUGR (131–133).

Implantation stimulates uterine stromal cells to proliferate and differentiate in response to progesterone. Decidualized uterine stromal cells acquire a unique secretory phenotype, which facilitates deep trophoblast invasion and remodeling of the maternal uterine stroma to form the maternal portion of the placenta, the decidua. Inhibiting formation of the decidua by deletion of key genes such as progesterone receptor (*Pgr*), *Bmp2* or *Hoxa11* abrogates the maintenance of pregnancy (134–137), and impaired growth of decidual tissue limits the size of the placenta. As such, defects which attenuate the decidualization response lead to the development of a small placenta with limited capacity to nourish the fetus, such as in mice with uterine-specific deletion of the TGF-β family receptor *Bmpr2* (138). Decidualization and formation of the decidua is dependent on the action of progesterone in an estrogen-primed uterus. Uterine stromal cells in the receptive endometrium express high levels of *Pgr* and its co-receptors including *Stat3*, which renders them competent to undergo decidualisation. Mice with deletion of *Pgr* or *Stat3* in the uterus are infertile as their progesterone resistance leads to complete failure of decidualization (135, 139). In recent studies into placental development with advanced maternal age in mice, it was shown that PGR and pSTAT3 levels are reduced in the uterine stroma of aged females, thus decreasing the ability of the endometrium to respond to progesterone (140, 141). This causes a blunted and delayed decidualization response with reduced proliferation and differentiation of uterine stromal cells, thus retarding the development of the decidua by up to 2 days in aged pregnancies. Such maternal-age related defects in the decidua impact on development of the trophoblast compartment, such that placentas from aged pregnancies often exhibit a small, malformed labyrinth and increased numbers of TGCs due to abnormal and skewed trophoblast differentiation. This is likely the result of dysregulation of the reciprocal signaling between the abnormal decidua and the trophoblast compartments. Other studies have similarly shown that defective decidualization has a knock-on effect on development of the trophoblast. Decidualization defects stemming from the maternal uterus can lead to reduced thickness of the labyrinth and SpT layers, with defects in GC differentiation and localisation (142–144), and increased differentiation into the TGC lineage (138, 143–146). These defects are likely to have a significant impact on placental function and fetal growth. Most strikingly, transfer of embryos from aged females into a young uterine environment rescues the placentation defects as well as embryonic growth and development, thus unequivocally attributing the effect to the mother and not the fetus (141). This is as of yet an understudied area in human pregnancy. Whilst abnormal decidualization with advanced maternal age has not yet been shown in humans, older women are at increased risk of pregnancy complications including fetal growth restriction and pre-eclampsia. The findings in the mouse suggest that this may be a result of defective decidualization due to endometrial dysfunction.

Abnormal decidualization can be associated with shallow invasion and inadequate transformation of the maternal vasculature, which is thought to be a causative factor and defining feature of obstetric complications such as pre-eclampsia and fetal growth restriction. In the mouse, deletion of the PI3K antagonist *Pten* in the decidua causes the development of an abnormally thick decidual compartment, due to decreased levels of apoptosis. The persistence of a thick decidual layer impedes trophoblast invasion and delays the remodeling of maternal blood vessels. This presumably limits the maternal blood supply to the placenta in early gestation, thus restricting fetal growth (142). Expression of a dominant negative form of the gap junction protein connexin-43 (*Gja1*) increases the expression of angiogenic factors *Flt1* and *Vegfa*, thus altering decidual angiogenesis. As a consequence, maternal blood spaces are disorganized and abnormally dilated, thus decreasing the surface area available for exchange (147). Conversely, deletion of the TGF-β family receptor *Bmpr2* in the uterus impairs decidual angiogenesis, resulting in a hypo-vascularised, under-perfused decidua with a poor capacity to nourish the fetus. This leads to reduced fetal growth and a miscarriage-like phenotype with progressive hemorrhaging of the placenta (138). These examples highlight the maternal impact on placentation and fetal growth as a consequence.

NEW INSIGHTS INTO MOLECULAR PATHWAYS CONTRIBUTING TO PLACENTAL DEVELOPMENT

Based on all the genetic and histopathological evidence summarized in the examples outlined above, it is clear that the placenta plays a pivotal role in the etiology of IUGR. With this in mind, the insights from a recent consortium study termed "Deciphering the Mechanisms of Developmental Disorders" (DMDD) is of key importance (148). This initiative aimed at systematically examining the impact of embryonic lethal or sub-viable gene mutations on embryonic and placental development in the mouse. It revealed that the placenta is far more frequently affected in these mutants than previously thought. Indeed, almost 70% of the mutant mouse lines examined that do not produce viable offspring at weaning exhibit a placental phenotype, a number that far exceeds the ∼10% placentation defects estimated on the basis of published data deposited in the Mouse Genome Informatics (MGI) database (56). Even though this study was conducted on mutants that do not produce viable offspring at the expected Mendelian ratios post-weaning, the vast majority of these mutants is also affected by IUGR prior to demise. Thus, these insights are highly valuable as it is conceivable that hypomorphic mutations in such genes may contribute to the spectrum of IUGR pathologies in viable offspring.

The key realization that most embryonic lethal or subviable mouse mutations will also suffer from placentation defects is of great impact. With some ∼5000 genes causing embryonic lethality, and an estimated two-thirds of these being associated

with placental defects, there are literally hundreds if not thousands of genes that remain unknown for their role in placental development. At least in some instances, these placentation defects will be causative of the developmental retardation and embryonic lethal phenotype. Even with the relatively limited number of genes analyzed as part of DMDD, it was possible to determine functional gene "hubs," i.e., clusters of physically or functionally interacting factors that very likely play pivotal roles in normal placentation. One example highlighted in that study was the Polycomb group gene *L3mbtl2*. Further mining of the DMDD database also reveals, for instance, the glycosylphosphatidylinositol (GPI)-anchor biosynthesis pathway as an important player in formation of the early labyrinth. Thus, efforts to identify embryonic lethal and subviable mutants are of immense value to identify novel genes, and functional protein complexes, that are important for normal placental development and hence also likely involved in the etiology of IUGR.

Data Mining Approaches

Apart from providing a first systematic survey of placental pathologies in embryonic lethal mouse mutants, another key advantage afforded by the DMDD study is the parallel analysis of both embryo and placenta for all mutant lines, allowing statistical co-association analyses of phenotype correlations. This analysis revealed a strong link between placental defects and heart, brain, and vascular abnormalities. These co-associations are not entirely unexpected, as a "heart-placenta axis" that describes an inter-dependence in the development of both organ systems has previously been suggested (78). However, this concept has so far been based on the analysis of very few, select genes only. The clear-cut nature of these developmental correlations in an entirely unbiased survey was hence compelling. They may indeed prove of great benefit for hypothesis-generating approaches to identify novel candidates for functional analysis in placental development. Thus, gene mutations that affect embryonic viability as well as heart, brain, or vascular development should enrich for factors that are also involved in normal placentation.

Underpinning the validity of this rationale on the above example of the GPI-anchor biosynthesis pathway, mutations in human *DPM1* and *PGAP2*, two pivotal genes implicated in placentation failure in the mouse, cause congenital disorders in which severe neurological dysmorphologies are a common feature (149, 150). Similarly, mutations in human *PIGL*, another placental regulator and GPI anchor synthesis protein identified by the DMDD study, are linked to a general developmental delay causing congenital heart disease, ocular colobomas, ichthyosiform dermatosis, mental retardation, ear anomalies and epilepsy (CHIME) syndrome (151). The fact that deletion of these genes in the mouse induces a severe trophoblast phenotype suggests an essential role of the GPI pathway in regulating early placentation linked to heart and neural development in the fetus.

Following this line of thought, we here screened the MGI database for the mammalian phenotype term "embryonic growth retardation" (MP: 0003984). This revealed 505 gene mutations, of which 54.7% (276) are also known to exhibit placental abnormalities (**Figure 4A**). Intriguingly, the remaining 229 lines in which a placental phenotype remains unknown show a significant prevalence of cardiovascular system phenotypes (51.1%), brain development defects (33.2%), or both (21.4 %) (**Figure 4B**). Therefore, it is tempting to speculate that at least some of these gene mutations will also display placentation defects.

To take this analysis even further we screened the genes associated with cardiovascular and/or brain phenotypes for expression in embryonic (ESCs) and trophoblast (TSCs) stem cells. In doing so we uncovered several genes that cluster into functional networks with an enriched expression in the trophoblast compartment compared to ESCs, thus suggesting a likely role in placentation (**Figure 4C**). One such node, identified within the cardiovascular phenotype gene collection, centers around a hydroxyacyl-CoA dehydrogenase complex (*Hadha*) gene. *Hadha* encodes the alpha subunit of the mitochondrial trifunctional protein complex involved in the mitochondrial beta-oxidation of fatty acids. The gene, as well as its known interactors, shows increased expression in TSCs compared to ESCs, reinforcing the notion of the likely functionality of this complex in trophoblast differentiation. Along similar lines, the *Coq7* gene encoding the enzyme demethoxyubiquinone monooxygenase was identified in the overlap between search terms "embryonic growth retardation" and "abnormal brain morphology." COQ7 is essential for ubiquinone biosynthesis. Ubiquinone acts as a cofactor during several redox processes like mitochondrial respiration. *Coq7*-deficient mice are embryonic lethal during mid-gestation with impaired neurogenesis and mitochondrial defects (152, 153). Although placental defects have not been described to date, *Coq7* is highly expressed in TSCs and placental trophoblast. Recently, a mutation of *COQ7* has been reported in a pregnancy complicated by oligohydramniosis, fetal lung hypoplasia, and growth retardation (154).

Finally, the joint overlap between search terms embryonic growth retardation, abnormal brain morphology and heart morphology reveals a number of genes that are highly expressed in TSCs, for example the muscle segment homeobox 2 (*Msx2*) transcription factor. *Msx1/2*-deficient mice show severe defects in cardiovascular and brain development and are growth-retarded (155). Our data would suggest that this mutant is likely to also exhibit defects in placental development. In fact, recent reports demonstrated that MSX2 is expressed in the human placenta and may regulate human trophoblast invasion (156). Another tangible example is the Hippo pathway component centered around *Lats2* (**Figure 4C**), that is known for its function in establishing cell polarity in the early embryo and the acquisition of trophoblast cell fate (15, 157). The phosphorylation state of YAP1 in particular is known as the broker between "inner" and "outer" cells in the morula-stage embryo, with unphosphorylated nuclear Yap1 being essential to induce trophoblast identity. The cytoskeletal protein Ezrin (*Ezr*) is a core hallmark of microvilli in epithelial cells and is strongly expressed in trophectoderm and its derivatives.

These various examples highlight the value of mining available large-scale datasets to identify novel candidate factors

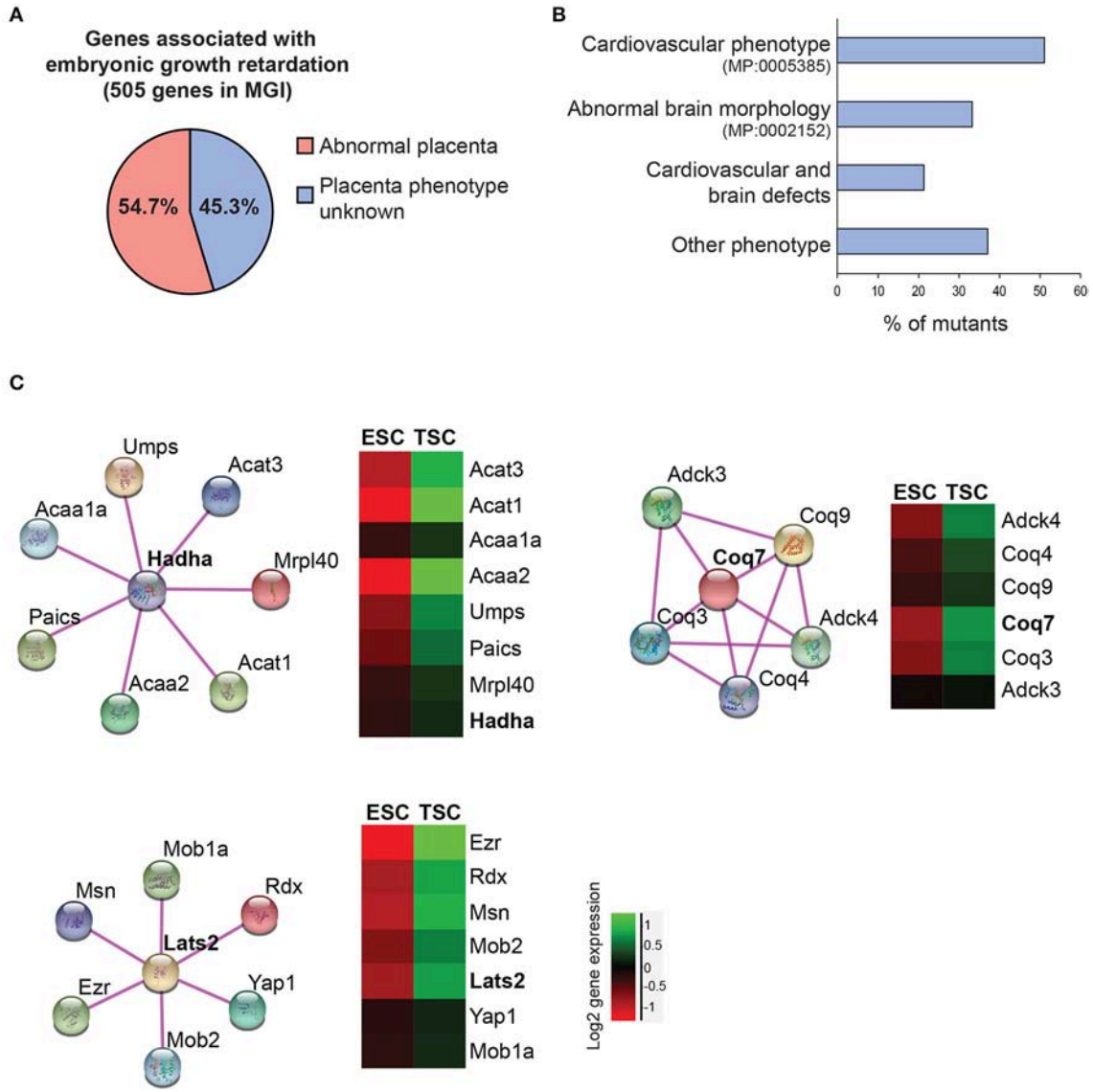

FIGURE 4 | Data mining approaches to identify molecular networks of potential importance in placental development. **(A)** Pie chart of genes extracted from the Mouse Genome Informatics database (www.informatics.jax.org) that are associated with embryonic growth retardation (MP: 0003984; 505 genes in total) and that are either scored as having an abnormal placenta (MP:0002086 or MP:0004264) or an unknown placental phenotype. **(B)** Breakdown of the proportion of mutants with unknown placental phenotype that also exhibit defects in heart and/or brain development. Since placental defects are often co-associated with cardiovascular and brain abnormalities (56), this selection will enrich for genes that have a function in the placenta. **(C)** Molecular network analysis using String (https://string-db.org/) to identify physical and/or functional gene interactions for selected examples, and expression levels of the identified complex components in trophoblast stem cells (TSCs) compared to embryonic stem cells (ESCs). The network components are enriched in the trophoblast compartment, arguing for a potential function in placental development.

and pathways that may be required for normal placentation. Once identified, these putative placental hubs require in-depth phenotypic and functional screening to confirm their involvement in extra-embryonic development. Nevertheless, they may prove powerful to accelerate the much-needed efforts in gaining a broader understanding of the collection of genes involved in placental biology both in healthy and pathological conditions.

CONCLUSIONS AND OUTLOOK

Perhaps the most striking outcome of the recent DMDD mouse phenotyping screen is the realization of the extent to which the number of genes contributing to placental development has been underestimated. The above examples provide a starting point on how these new and extensive datasets can be mined in the future with the aim of identifying novel molecular networks that may

have key functions during placentation. Even within the small set of genes highlighted here, it is clear that some have already been linked to human placentation and/or developmental problems. These links inspire with confidence that the combination of mouse phenotyping data, gene expression profiles, physical, and/or functional protein interaction networks and human disease links are indeed powerful approaches to identify such functional gene clusters. Arguably, rather than individual genes, the functionality of larger protein complexes and/or gene networks is more likely to be conserved between species, making this approach more powerful and more promising for translational studies. Once confirmed for an involvement in the etiology of placental defects, it is conceivable that such genes may become part of mutation screening panels for refined diagnosis and may constitute targets for drug development to improve our understanding and potential treatment options of IUGR in the future. Overall, the depth and molecular detail of the analysis of recent mouse mutants has opened up many new avenues for research aimed at understanding the molecular basis of normal placentation and of placental pathologies in humans.

AUTHOR CONTRIBUTIONS

LW, VP-G, and MH contributed to the conceptualisation and writing of the manuscript. MH assembled **Figure 1**, LW **Figures 2**, **3**, and VP-G **Figure 4** and **Supplementary Table 1**.

ACKNOWLEDGMENTS

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC), and by a Ph.D. studentship from the Medical Research Council (MRC) to LW. VP-G is recipient of a Next Generation Fellowship from the Centre for Trophoblast Research, University of Cambridge, UK.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00570/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 16 November 2018
doi: 10.3389/fphys.2018.01583

Brain Volumes and Developmental Outcome in Childhood Following Fetal Growth Restriction Leading to Very Preterm Birth

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Background: Children born very preterm (PT) after fetal growth restriction (FGR) exhibit cognitive impairment at early school age. The relationship between neurodevelopmental impairment and attained regional brain volumes is unknown.

Methods: We studied 23 preterm children with FGR (PT-FGR), 24 matched preterm children AGA (PT-AGA), and 27 matched term AGA children (T-AGA) by measuring brain volumes with magnetic resonance imaging at early school age. Cognitive and motor functions were assessed by the Wechsler Intelligence Scales for Children and the ABC-Movement score.

Results: The mean (*SD*) full-scale IQ was 80 (17) in the PT-FGR group and 103 (12) in the PT-AGA group ($p < 0.001$). The PT-FGR group had lower mean total, gray matter, white matter, thalamic, cerebellar white matter, and hippocampal volumes as compared to the T-AGA group ($p = 0.01$, 0.04, 0.003, 0.002, 0.001, and 0.009, respectively). Brain volumes did not differ significantly between the PT groups. Reduction of hippocampal volume correlated with degree of growth restriction at birth ($r = 0.46$, $p = 0.05$). Neither the full-scale IQ nor the ABC movement score <5th percentile were related to brain volumes.

Conclusion: Brain volumes as determined by MRI at early school age were primarily associated with degree of prematurity at birth and less with FGR. Regional brain volumes did not discriminate cognitive and motor function beyond that predicted by gestational age at birth.

Keywords: brain volumes, magnetic resonance imaging, neuro-development, preterm birth, fetal growth restriction

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *01 August 2018*
Accepted: *23 October 2018*
Published: *16 November 2018*

Citation:
Morsing E, Malova M, Kahn A, Lätt J, Björkman-Burtscher IM, Maršál K and Ley D (2018) Brain Volumes and Developmental Outcome in Childhood Following Fetal Growth Restriction Leading to Very Preterm Birth. Front. Physiol. 9:1583. doi: 10.3389/fphys.2018.01583

Abbreviations: ADD, Attention deficit disorder; AGA, Appropriate for gestational age; ARED, Absent or reversed end-diastolic; BPD, Bronchopulmonary dysplasia; FGR, Fetal growth restriction; FIQ, Full scale IQ; GA, Gestational age; GM, Gray matter; MRI, Magnetic resonance imaging; PIQ, Performance IQ; PT, Preterm; ROP, Retinopathy of prematurity; ROI, Regions of interest; T, Term; VIQ, Verbal IQ; WM, White matter.

INTRODUCTION

Fetal growth restriction (FGR) at early GA is associated with high risk of perinatal mortality, morbidity, and long-term neurodevelopmental impairment (Torrance et al., 2010; Baschat, 2011). ARED blood flow in the umbilical artery assessed by Doppler velocimetry is associated with adverse outcome in preterm FGR (PT-FGR) fetuses (Hartung et al., 2005). No consensus on clinical management of FGR fetuses diagnosed in the second trimester (early-onset) has been achieved so far (Baschat and Odibo, 2011). Prenatal clinical management has to consider the high risk of neonatal complications associated with severe prematurity against the substantial risk of fetal death.

Previous studies, with the objective of relating impaired fetal growth to different aspects of outcome have mainly used birthweight small for GA, (SGA) as a proxy and a common denominator for FGR. As well known, this definition is imprecise and will include constitutionally small infants as well as truly growth-retarded infants. The objective of the present study is to evaluate long-term effects of FGR leading to very preterm birth (<30 GW). The patho-physiological consequences of early-onset FGR, i.e., leading to very preterm birth, are sensitively detected by fetal blood flow evaluation using Doppler ultrasound (Figueras and Gratacos, 2014).

Infants born very preterm (PT) were reported to exhibit reduction in total and regional cerebral volumes determined by MRI (Keunen et al., 2012) and the observed brain volume reduction has been shown to persist into childhood and adolescence (Reiss et al., 2004; Northam et al., 2011). In fetuses affected by late-onset growth restriction, fetal MRI showed different cortical development as compared to fetuses with normal growth (Egana-Ugrinovic et al., 2013). Subjects with late-onset FGR, as determined by birthweight small for GA and born at term age, exhibited a global reduction in brain volume as well as regional reductions in cortical surface area (Ostgard et al., 2014).

Fetal growth restriction leading to very preterm birth, i.e., early-onset FGR, has been associated with altered brain structure and lower brain volumes during the PT period and at term-equivalent age (Tolsa et al., 2004; Thompson et al., 2007). Interpretation of previous studies on FGR and brain volumes is complicated by the heterogeneity of study designs and inclusion criteria. Some authors have described reduced brain volumes in small for GA subjects born at late gestation with no information on preceding fetal blood flow examinations (Ostgard et al., 2014). Differences in age of subjects at MRI assessment also complicate comparisons of brain volumes studies, since brain growth changes with age in healthy subjects (Sussman et al., 2016).

To date, there are no controlled studies investigating the effect of FGR leading to very preterm birth on MRI-assessed regional brain volumes in children; thus, it is unclear whether the alterations in brain volumes persist into childhood. Infants with FGR and ARED flow leading to very preterm birth have an increased neonatal morbidity and an adverse cognitive outcome at school age (Brodszki et al., 2009; Morsing et al., 2011). Clinical guide-lines for management of this group of fetuses still differ between centers and countries. It is therefore essential, from a neuro-scientific as from a clinical point of view, to investigate brain development in this well-defined group of subjects. The aim of this study was to evaluate regional brain volumes and their relationship to cognitive and motor outcome at 8 years of age in children born very preterm after FGR and ARED umbilical artery blood flow.

PATIENTS AND METHODS

Population

The present work is part of a prospective case-control follow-up study of children born very preterm (<30 gestational weeks) after early-onset FGR and ARED blood flow in the umbilical artery. Doppler flow velocity signals from the umbilical artery, the middle cerebral artery, DV, umbilical veins and from both maternal uterine arteries were recorded using Philips HDI 5000 or Philips IU22 (Philips Medical Systems, Bothell, WA, United States) ultrasound systems. Abnormal DV flow was defined as absent or reverse flow during the a-wave. The Doppler velocimetry was in all cases performed in a standardized way by experienced sonographers. FHR recordings and Doppler velocimetry were performed daily. Between 1998 and 2004, 42 such live-born neonates were delivered on fetal indication at the level III perinatal center at Skane University Hospital in Lund, Sweden. Since 1998, a proactive clinical management protocol is used for management of very preterm fetuses with FGR aiming to avoid severe fetal hypoxia and deterioration of fetal condition. The protocol indicates delivery at the occurrence of reversed end-diastolic (RED) flow in the umbilical artery, and/or if there are rapidly progressing changes in the ductus venosus (DV) blood velocity waveform or pathological changes of fetal heart rate (FHR; loss of variability, late decelerations). After 26 gestational weeks, absent end-diastolic (AED) flow in the umbilical artery was indication for delivery after the full course of antenatal steroid treatment. The clinical protocol included delivery by cesarean section and neonatal active care comprising early surfactant treatment and early enteral feeding with human milk. During the same period, two control groups with birth weight AGA were identified; one group consisted of preterm infants matched for GA and sex (PT-AGA), and the other group was born at term matched for sex and age at examination (T-AGA). At the age of 7–8 years, follow-up examinations were performed. Perinatal data, prevalence of cerebral palsy, as well as neurocognitive, cardiovascular and pulmonary outcome in this cohort have been reported previously (Brodszki et al., 2009; Morsing et al., 2011, 2012, 2014).

Children from the three groups that underwent an examination with brain MRI at 8 years of age constituted the index group (PT-FGR, n = 23) and two control groups (PT-AGA, n = 24; T-AGA, n = 27) of the present study.

Movement ABC

Children were evaluated with The Movement Assessments Battery for Children 2nd edition (Movement ABC) consisting of eight items divided into three subtests: manual dexterity,

ball skills, and static/dynamic balance. (Henderson and Barnett, 2007) High scores denote motor performance deficits. Scores below the 5th percentile indicate severe motor impairment, and scores between the 5th and 15th percentile indicate minor motor impairment.

Cognitive Tests and Behavior Questionnaire

Cognitive evaluation was performed by Wechsler scales (the Wechsler Preschool and Primary Scale of Intelligence-III and the Wechsler Intelligence Scale for Children-III, 1991 revision, British version) at 5–8 years of age (range 60–105 months). (Wechsler, 1991, 2002; Brown, 2001) Both tests consist of two IQ subscales, VIQ and PIQ, forming the full-scale IQ (FIQ). All scales have a mean of 100 points and *SD* of 15. Cognitive impairment was defined as FIQ < 70 (> 2 *SD* below the normative mean).

During follow-up visit, the parents were interviewed and filled in two scoring questionnaires regarding attention-deficit disorder (ADD; Brown's ADD scales)(Brown, 2001) and behavior problems (Strengths and Difficulties Questionnaire (SDQ). Brown's ADD consists of 44 items and includes tasks examining ability to sustain attention and energy, effort to complete tasks, to regulate moods and recall learned material. A score >55 indicates risk for ADD. The SDQ is a behavioral screening questionnaire that comprises 25 items divided into subscales (prosocial, hyperactivity, emotional problems, conduct, and peer problems). A total score of 13 was considered to be borderline or high.

MRI Scanning

All children were informed about the MRI procedure beforehand and were awake during the scanning. None of the children were sedated. Ear plugs and headphones were used for hearing protection and children could watch a movie during the examination by means of projection on a screen behind the scanner and viewed through a mirror mounted to the head coil.

The MRI investigations were performed on a 3T scanner (Achieva, Philips, Best, Netherlands) with an eight channel SENSE head coil. Volumetric data were acquired using a T1 3D magnetization prepared rapid acquisition with gradient echo (MPRAGE) sequence with isotropic voxel size of 1 mm^3, allowing reconstruction in any plane, repetition time = 9 ms, echo time = 4 ms, and flip angle = 10°. Reconstruction and segmentation of the brain was performed with the FreeSurfer image analysis suite[1]. Volumetric data were acquired for total intracranial volume, GM, WM, cerebrospinal fluid, cerebellar GM and WM, and thalamus. All segmentations were subsequently reviewed and, if necessary, the automatically segmented volumes were adjusted manually using in-house MatLab (Mathworks, MA, United States) based software. In particular, the anterior border of the cerebellar WM was adjusted to consequently exclude brainstem areas. The peduncles of the cerebellum were included in the WM volumes and the vermis was included in the GM volumes.

[1]http://surfer.nmr.mgh.harvard.edu

Hippocampal segmentation was performed manually on a PACS workstation (IDS7, Sectra, Linköping, Sweden) in consensus by two radiologists blinded to perinatal data. T1 MPRAGE images were separately for both sides reformatted to an oblique coronal viewing plane perpendicular to the long axis and in parallel to the anterior and posterior limits of the hippocampus. Delineation was performed in a posterior to anterior direction with the plane showing the crus of the fornix defining the level of the most posterior aspect of the hippocampus and the plane where the temporal horn appears medially and beneath the amygdala the most anterior portion. As further boundaries of the hippocampus we used the alveus (superior), WM of the para-hippocampal gyrus (inferior), the temporal horn of the lateral ventricle (lateral), and the ambient cistern (medial). The subiculum was included in the hippocampus. Anteriorly the temporal horn of the lateral ventricle and the alveus were used to separate hippocampus from amygdala. When in doubt, an imaginary line between the ambient cistern and the middle of the temporal horn of the lateral ventricle was used. ROIs were first drawn by observer 1 according to the protocol, then observer 2, in a separate session defined visually the boundaries of the hippocampi on unmarked projections ant then checked ROIs for mismatches. All suggested changes of hippocampal delineation were discussed in consensus between the observers and then implemented.

Data Collection and Analysis

Demographic data and clinical parameters, including prenatal, perinatal, and neonatal data were collected from the obstetric and pediatric patient records. Head circumference was measured by a pediatrician at the follow-up examination. Information on socioeconomic factors was obtained from questionnaires administered to the parents while the children attended the tests. Cognitive, motor, and behavioral test results, as well as MRI data were registered. Possible associations between the volume of brain structures, clinical data and neurobehavioral test results were assessed.

Statistical analyses were performed using SPSS 23.0 statistical software (SPSS Inc, Chicago, IL, United States). Categorical variables were compared between groups by the χ^2 test. Differences in continuous variables were assessed with two-way analysis of variance (ANOVA) with *post hoc* Bonferroni correction for multiple group comparisons. *P*-values of <0.05 were considered statistically significant. Confounders were explored by linear regression analysis.

RESULTS

Perinatal Clinical Data and Postnatal Morbidity

Birth characteristics, neonatal morbidity, and rate of cerebral palsy in the three groups are presented in **Table 1**. All 23 infants of the index group had ultrasonically estimated fetal weight more than 2 *SD* below the mean of the Swedish reference population (Marsal et al., 1996). Eighteen fetuses had absent, and five had reversed end-diastolic blood flow in the umbilical

TABLE 1 | Birth characteristics, neonatal morbidity, and rate of cerebral palsy.

				Significance of difference		
	PT-FGR (*n* = 23)	**PT-AGA (*n* = 24)**	**T-AGA (*n* = 27)**	**PT-FGR– PT-AGA**	**PT-FGR – T-AGA**	**PT-AGA – T-AGA**
Gestational age weeks, mean ± SD	26.4 ± 1.5	26.7 ± 1.6	39.4 ± 0.7	*ns*	*<0.001*	*<0.001*
ARED flow in the umbilical artery, *n* (%)	23 (100)	0	0	*<0.001*	*<0.001*	*na*
Birthweight g, median (range)	664 (395–976)	1032 (660–1790)	3550 (3000–4390)	*<0.001*	*<0.001*	*<0.001*
Birthweight deviation %, median (range)	−37 (−63 – −23)	−5 (−22 –+14)	−1 (−17 –+29)	*<0.001*	*<0.001*	*0.05*
Apgar score < 7 at 5 min *n* (%)	1 (4)	4 (17)	0	*ns*		
Cesarean section *n* (%)	23 (100)	14 (58)	0	*<0.001*		
Sex, female/male *n*	13/10	11/13	14/13	*ns*	*ns*	*ns*
Term head circumference cm, mean ± SD	34.4 ± 1.5	35.6 ± 1.0	35.5 ± 1.2	*0.02*	*0.02*	*ns*
IVH III/PVHI *n* (%)	1 (4)	2 (8)	0	*ns*		
ROP *n* (%)	4 (17)	6 (25)	0	*ns*		
NEC *n* (%)	3 (13)	0	0	*ns*		
BPD *n* (%)	16 (70)	6 (25)	0	*0.003*		
Postnatal steroid treatment *n* (%)	9 (39)	5 (21)	0	*ns*		
Septicemia *n* (%)	14 (61)	6 (25)	0	0.02		
Cerebral palsy *n* (%)	2 (9)	4 (17)	0	*ns*		

PT, preterm; T, term; AGA, appropriate for gestational age; birthweight deviation % of expected birthweight according to Swedish standard; FGR, fetal growth restriction; ARED, absent or reversed end-diastolic blood flow; ns, non-significant; IVH, Intraventricular hemorrhage; PVHI, peri-ventricular hemorrhagic infarction; ROP, retinopathy of prematurity; NEC, necrotizing enterocolitis; BPD, bronchopulmonary dysplasia.

artery. Fifteen fetuses out of 23 had signs of brain-sparing, i.e., middle cerebral artery pulsatility index >2 *SD* below the GA related mean (Mari and Deter, 1992). The mean (*SD*) GA at birth was 26.4 (1.5) weeks and all infants were small-for-GA with birthweight < mean−2 *SD* of the reference (Marsal et al., 1996).

Infants in the control groups (PT-AGA and T-AGA) had AGA birth weight. Antenatal Doppler velocimetry was not performed in the control children.

The Apgar score <7 at 5 min and sex distribution were similar in the preterm groups. Prevalence of severe intraventricular hemorrhage, ROP and necrotizing enterocolitis, as well as use of postnatal steroids was similar in the preterm groups. The PT-FGR group had higher rate of septicemia and BPD than the control PT-AGA group.

Cognitive Function, Motor Performance, and Behavior

Results of cognitive evaluation, motor performance, ADD, and behavior are shown in **Table 2**. The PT-FGR group had lower motor performance score, higher scores in attention deficit and behavior questionnaires compared to the T-AGA group. Scores from Brown's ADD and SDQ scales did not differ between the two preterm groups. PIQ and FIQ were lower in both preterm groups compared to the T-AGA group. FIQ was lower in the PT-FGR group than in the PT-AGA group.

Subjects Not Examined With MRI

The numbers of subjects evaluated for cognitive and behavioral outcome at 6–8 years of age in the background population (Morsing et al., 2011) but not examined by MRI were 11, 10, and 7 in the background PT-FGR, PT-AGA, and the T-AGA groups, respectively. Birthweight deviation and GA at birth, as well as the cognitive outcome, behavior, and motor performance did not differ between the infants examined and those not examined with MRI.

MRI Brain Volumes

The PT-FGR group had significantly lower mean total intracranial, GM and WM, thalamic, cerebellar WM and hippocampal volumes as compared to the T-AGA group (**Table 3** and **Figure 1**). The PT-AGA group had lower mean WM, thalamic and cerebellar WM volumes as compared to the T-AGA group. Brain volumes did not differ significantly between the PT-FGR and the PT-AGA groups (**Table 3** and **Figure 1**). When compared to term controls, female FGR subjects had smaller thalamic, cerebellar WM and hippocampal volumes, while males had smaller WM volumes.

Group differences were also evaluated for regional brain volumes in relation to total intracranial volume. Relative cerebellar WM volume was lower in the PT-FGR and the PT-AGA groups as compared to the T-AGA group, $p = 0.016$ and

TABLE 2 | Cognitive evaluation by WISC-III/WPPSI-III, motor performance by ABC-movement, attention deficit disorder and behavior by Brown's ADD and SDQ and measurements of head circumference at 5–8 years.

				Significance of difference		
	PT-FGR (*n* = 23)	**PT-AGA (*n* = 24)**	**T-AGA (*n* = 27)**	**PT-FGR – PT-AGA**	**PT-FGR – T-AGA**	**PT-AGA – T-AGA**
Parental education						
High school/university						
Mother, *n* (%)	9/23 (39)	12/23 (52)	14/26 (54)	*ns*	*ns*	*ns*
Father, *n* (%)	5/23 (22)	8/23 (35)	11/26 (42)	*ns*	*ns*	*ns*
Verbal IQ mean ± SD	85 ± 18	95 ± 14	102 ± 11	*ns*	*<0.001*	*ns*
Performance IQ mean ± SD	80 ± 16	87 ± 15	105 ± 15	*ns*	*<0.001*	*<0.001*
Full scale IQ mean ± SD	80 ± 17	90 ± 13	103 ± 12	*0.05*	*<0.001*	*0.003*
Motor performance < 5th percentile, *n* (%)	8 (34)	3 (12)	1 (4)	*ns*	*0.002*	*ns*
ADD combined mean ± SD	51.4 ± 10.9	48.1 ± 10.1	42.1 ± 5.6	*ns*	*0.002*	*ns*
ADD combined > 55, *n* (%)	8 (35)	7 (29)	1 (4)	*ns*	*0.007*	*0.019*
SDQ total, mean ± SD	9.6 ± 5.1	7.2 ± 5.9	4.4 ± 4.9	*ns*	*<0.001*	*ns*
SDQ total > 13, *n* (%)	6 (26)	4 (17)	1 (4)	*ns*	*0.04*	*ns*
Head circumference at MRI, cm, mean ± SD	51.7 ± 1.6	52.9 ± 1.6	54.6 ± 1.6	*ns*	*<0.001*	*0.009*

WISC, Wechsler Intelligence Scale for Children; 3rd edition. WPPSI, Wechsler Preschool and Primary Scale of Intelligence; 3rd edition. ABC-movement, Movement assessments battery for children; Brown's ADD, attention deficit disorder; SDQ, strength and difficulties questionnaire; PT, preterm; AGA, appropriate for gestational age; FGR, fetal growth restriction; MRI, magnetic resonance imaging.

0.033, respectively. Remaining regional brain volumes in relation to total intracranial volume exhibited no differences between groups.

After adjustment for GA at birth, head circumference at term age correlated positively with global as well as with regional brain volumes within the respective PT groups. Fetal brain sparing was not associated with brain volumes. Weight deviation at birth was positively correlated to all regional brain volumes, but when GA at birth and sex were taken into account, only hippocampal volume remained significantly associated ($r = 0.46$, $p = 0.05$).

The presence of neonatal risk factors with a potential impact on brain growth, such as severe intraventricular hemorrhage grade III or periventricular hemorrhagic infarction, septicemia, and necrotizing enterocolitis, was not related to brain volumes. In univariate analysis, BPD was significantly associated with decreased volumes of GM, WM and cerebellar WM, but after adjustment for GA, birth weight deviation at birth and sex only GM volumes remained associated with BPD ($r = 0.43$, $p = 0.01$). Any stage of ROP was significantly associated with decreased volumes of WM, thalamus, and cerebellar GM, but after adjustment for GA, weight deviation at birth and sex, ROP remained significantly associated with cerebellar GM ($r = 0.60$, $p = 0.02$). Postnatal steroid treatment with betamethasone did not correlate with brain volumes.

Motor performance, cognitive outcome, behavior, and ADD were not associated with differences in any of the measured brain volumes. Sex had no influence on the relationship between regional brain volumes and neurodevelopmental outcome.

DISCUSSION

In this prospective study, we examined neurodevelopmental outcome and brain growth as determined by brain MRI at early school age in children born very preterm after early-onset FGR, and in matched preterm and term AGA controls. The PT-FGR group had a higher rate of cognitive impairment than PT-AGA subjects, however, we did not observe any corresponding differences in global or regional brain volumes as determined by MRI.

All children in the PT-FGR group had ARED blood flow in the umbilical artery prior to delivery and were actively delivered before 30 gestational weeks in order to prevent further worsening of fetal distress. We have previously reported that neonatal mortality, cerebral morbidity, and rate of cerebral palsy at 2 years of age in the PT-FGR group were comparable to those of children delivered very preterm due to other indications (Brodszki et al., 2009). However, subsequent follow-up showed that cognitive impairment at early school age was more prevalent in the PT-FGR as compared to the PT-AGA group, mainly due to decreased cognitive performance in growth restricted boys (Morsing et al., 2011).

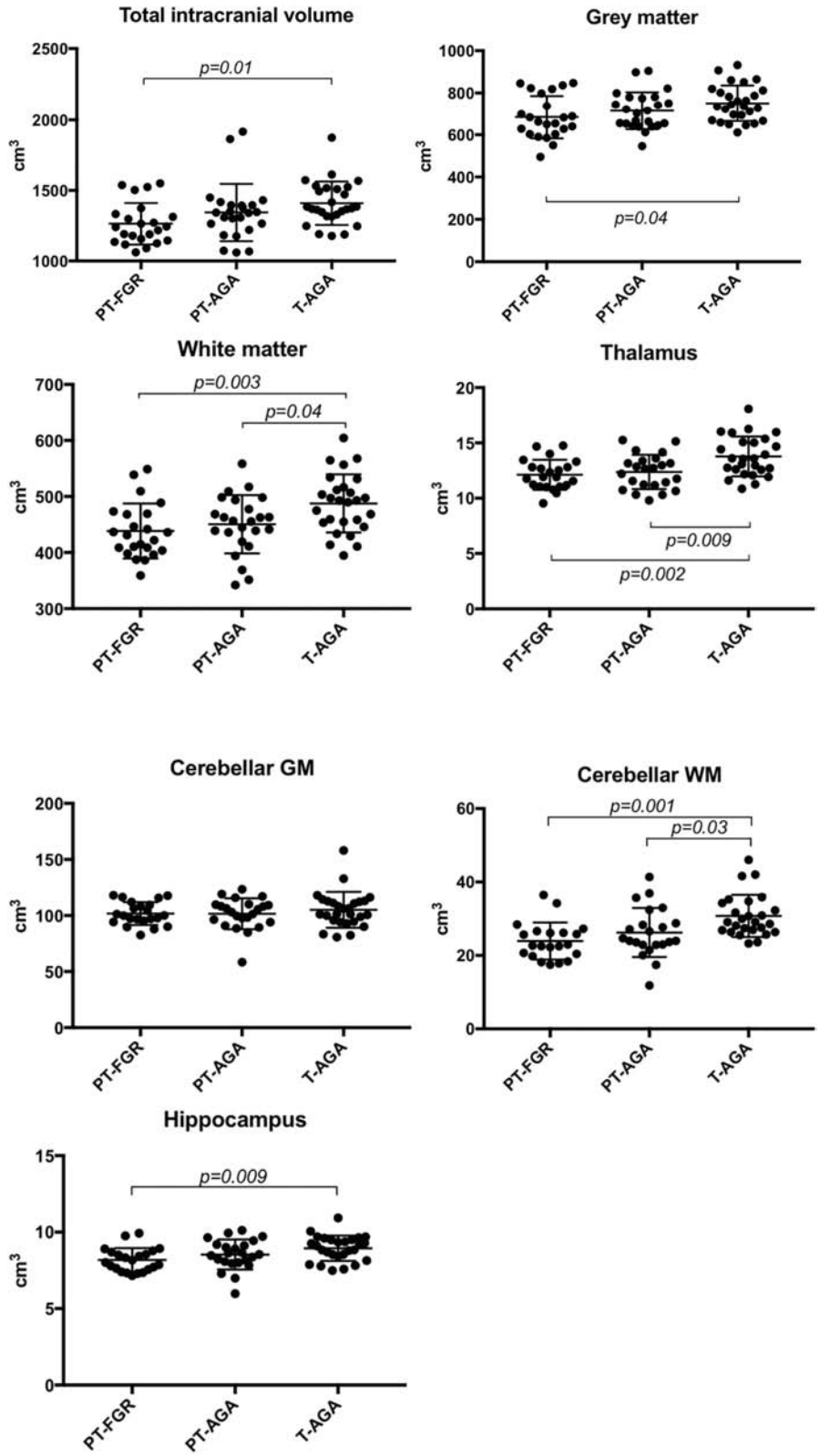

FIGURE 1 | Dot plots describing individual values of total intracranial brain volume and regional brain volumes in subjects with fetal growth restriction and very preterm birth (PT-FGR group), very preterm birth and birthweight appropriate for gestational age (PT-AGA group), and birthweight appropriate for GA at term age (T-AGA group). Horizontal lines depict group means and standard deviations. Cerebellar GM, cerebellar gray matter; Cerebellar WM, cerebellar white matter.

TABLE 3 | Brain volumes (cm^3, mean ± SD).

Brain structure	Group			Significance of difference		
cm^3	PT-FGR (*n* = 23)	PT-AGA (*n* = 24)	T-AGA (*n* = 27)	PT-FGR– PT-AGA	PT-FGR – T-AGA	PT-AGA – T-AGA
Total intracranial volume	1264 ± 147	1344 ± 203	1409 ± 153	*ns*	*0.01*	*ns*
male	1287 ± 153	1434 ± 213	1462 ± 179	*ns*	*ns*	*ns*
female	1246 ± 146	1237 ± 130	1361 ± 111	*ns*	*ns*	*ns*
Gray matter	684 ± 101	714 ± 88	750 ± 85	*ns*	*0.04*	*ns*
male	705 ± 119	747 ± 88	777 ± 102	*ns*	*ns*	*ns*
female	669 ± 87	676 ± 75	726 ± 59	*ns*	*ns*	*ns*
White matter	438 ± 49	450 ± 52	488 ± 52	*ns*	*0.003*	*0.04*
male	449 ± 52	471 ± 39	509 ± 50	*ns*	*0.02*	*ns*
female	431 ± 46	426 ± 55	468 ± 46	*ns*	*ns*	*ns*
Thalamus	12.1 ± 1.4	12.4 ± 1.5	13.8 ± 1.8	*ns*	*0.002*	*0.009*
male	12.4 ± 1.1	12.7 ± 1.8	14.1 ± 2.3	*ns*	*ns*	*ns*
female	11.9 ± 1.5	12.0 ± 1.3	13.5 ± 1.3	*ns*	*0.02*	*0.04*
Cerebellar GM	101.7 ± 10.2	101.5 ± 13.8	105.1 ± 15.9	*ns*	*ns*	*ns*
male	107.6 ± 9.7	108.9 ± 8.7	105.3 ± 21.1	*ns*	*ns*	*ns*
female	97.7 ± 8.8	93.6 ± 14.3	104.9 ± 9.7	*ns*	*ns*	*0.05*
Cerebellar WM	24.0 ± 4.9	26.0 ± 6.6	31.7 ± 9.1	*ns*	*0.001*	*0.03*
male	25.40 ± 6.4	28.3 ± 5.9	32.7 ± 10.1	*ns*	*ns*	*ns*
female	23.0 ± 3.0	23.5 ± 7.0	30.7 ± 8.2	*ns*	*0.02*	*0.03*
Hippocampus	8.2 ± 0.8	8.5 ± 1.0	8.9 ± 0.8	*ns*	*0.009*	*ns*
male	8.5 ± 0.9	8.8 ± 0.9	9.1 ± 0.9	*ns*	*ns*	*ns*
female	8.0 ± 0.6	8.3 ± 1.1	8.8 ± 0.7	*ns*	*0.04*	*ns*

ns, non-significant; GM, gray matter; WM, white matter.

Several studies have correlated adverse neurodevelopment with time of onset of growth restriction, (Baschat, 2014) severity of Doppler changes, (Schreuder et al., 2002) GA at delivery, (Baschat and Odibo, 2011), and neonatal morbidity (Yeh et al., 2004; Trittmann et al., 2013). The effect of fetal brain sparing was found to be associated with impaired cognitive outcome and to have negative effects on the brain (Scherjon et al., 2000). The majority of children in the present PT-FGR group had signs of fetal brain sparing, however, we did not find any relationship between redistribution of flow and brain volumes at early childhood.

The main result of the present study is the observation of smaller global and regional brain volumes in the preterm FGR group compared to the term AGA group, although no clear differences could be observed between the PT-FGR and PT-AGA groups. In previous studies, MRI performed at term-equivalent age (Tolsa et al., 2004) and at 12 months of age (Padilla et al., 2014) has shown reduced brain tissue volumes in infants born after FGR when compared to GA matched controls. It is important to note that subjects included in those studies had a considerably higher mean GA at birth, which, in absence of extreme prematurity, may increase the possibility of observing effects of FGR *per se*. The conflicting results might also reflect differences in pathophysiology between early-onset and late-onset FGR, and in the related clinical managements.

In our controlled study of preterm FGR subjects, hippocampal volume was positively correlated to weight deviation at birth. This is consistent with both experimental (Mallard et al., 2000) and clinical (Lodygensky et al., 2008) studies describing hippocampal vulnerability in growth restriction, possibly mediated by hypoxia and/or reduced supply of nutrients.

We observed sex-related differences in regional brain volumes in our study population. In comparison to the T-AGA reference group, female PT-FGR subjects had reduced thalamic, cerebellar, and hippocampal volumes, while male PT-FGR subjects had smaller WM volumes. These results are in accordance with literature that shows smaller WM volume at 8 years of age in preterm males (Reiss et al., 2004). Sex differences in brain morphology corresponding to those found in the present study were recently observed in a cohort of extremely preterm infants: female subjects had more abnormalities in the cerebellum and male subjects displayed delayed myelination (Skiold et al., 2014).

Among neonatal morbidities, BPD remained related to decreased GM volume after adjustment for GA and weight deviation at birth. The relationship between BPD and MRI abnormalities has been described previously. Brain abnormalities and delayed maturation in WM and thalamus were observed in MRI at term-equivalent age of PT infants with BPD. (Rose et al., 2014; Neubauer et al., 2015) In two other cohorts, BPD and exposure to postnatal dexamethasone resulted in smaller GM volume at term (Murphy et al., 2001) and in reduced total brain volume at adolescent age (Cheong et al., 2014). In our population, smaller GM volume was related to BPD but not to postnatal steroid treatment. The effect of postnatal treatment with betamethasone on brain growth has not been evaluated in

PT infants as opposed to that of dexamethasone (Cheong et al., 2014). Speculatively, betamethasone may be less harmful to the brain tissue.

Further, any stage of ROP was related to reductions in cerebellar cortex volumes and these relationships remained significant after adjustment for confounders. In a recent study, presence of ROP was associated with smaller biometric measurements and higher prevalence of brain abnormalities on term-equivalent age MRI (Naud et al., 2017). We recently reported a relationship between any stage of ROP and reduced WM and cerebellar volume at term age as determined by MRI (Sveinsdottir et al., 2018). These findings suggest that common pathways may lead to impaired neural and neurovascular development in the brain and retina.

The main strengths of the present study are its prospective design and careful matching of study groups. The study has several limitations. Fetal Doppler measurements were not performed in the control groups. However, all subjects in the control groups were AGA at birth, which suggests that fetal blood flow impairment was less probable. Further, the study and control groups were relatively small and conclusions have to be drawn with caution. Thirty per cent of the children/parents did not consent to MRI examination which further reduced the strength of the study. However, cognitive and motor performance outcomes did not differ between children who declined and those who performed the MRI assessment in our study. The latter observation is reassuring in view of the finding in a recent meta-analysis, reporting that a greater loss to follow-up was correlated to higher rates of neurodevelopmental impairment (Guillen et al., 2012).

It is still unclear to which extent FGR with hemodynamic impairment prior to very preterm birth modifies or accentuates the risks of prematurity. Contrary to other studies, our data on the FGR group delivered very preterm did not show smaller regional brain volumes compared to those of matched PT-AGA controls. Cognitive impairment, more prevalent in the FGR group, was not related to brain volumes. One can speculate that the reduced brain volumes are not the most important pathophysiological factor connected to functional outcomes. Alternatively, reduced brain volumes seen at early ages, i.e., in neonates and in infancy, may normalize with age. We are currently analyzing data from diffusion tensor imaging (DTI), performed in parallel with the MRI volumetric study. Data on WM structure may provide important additional information about the effect of FGR on brain development.

ETHICS STATEMENT

This study was approved by the Regional Research Ethics Committee at Lund University and examinations of the children were performed after their parents gave written informed consent.

AUTHOR CONTRIBUTIONS

EM conceptualized and designed the study, coordinated and supervised the data collection, carried out the statistical analysis, drafted the initial manuscript, and reviewed and revised the manuscript. KM, DL, and IB-B, conceptualized and designed the study, designed the data collection instruments, and critically reviewed and revised the manuscript. AK, MM, and JL, collected the data, carried out the initial analyses, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agreed to be accountable for all aspects of the work.

FUNDING

This study was supported by the Swedish Research Council, governmental ALF research grants to Lund University and Lund University Hospital and Queen Silvia Foundation for Medical Research.

ACKNOWLEDGMENTS

Gunborg Mattson, physiotherapist, B.Sc., who performed the ABC-movement and Malena Åsard, psychologist, who performed the psychological tests.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 19 November 2018
doi: 10.3389/fendo.2018.00671

Mechanisms Underpinning Adaptations in Placental Calcium Transport in Normal Mice and Those With Fetal Growth Restriction

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Fetal delivery of calcium, via the placenta, is crucial for appropriate skeletal mineralization. We have previously demonstrated that maternofetal calcium transport, per gram placenta, is increased in the placental specific insulin-like growth factor 2 knockout mouse (P0) model of fetal growth restriction (FGR) compared to wild type littermates (WTL). This effect was mirrored in wild-type (WT) mice comparing lightest vs. heaviest (LvH) placentas in a litter. In both models increased placental calcium transport was associated with normalization of fetal calcium content. Despite this adaptation being observed in small normal (WT), and small dysfunctional (P0) placentas, mechanisms underpinning these changes remain unknown. Parathyroid hormone-related protein (PTHrP), elevated in cord blood in FGR and known to stimulate plasma membrane calcium ATPase, might be important. We hypothesized that PTHrP expression would be increased in LvH WT placentas, and in P0 vs. WTL. We used calcium pathway-focused PCR arrays to assess whether mechanisms underpinning these adaptations in LvH WT placentas, and in P0 vs. WTL, were similar. PTHrP protein expression was not different between LvH WT placentas at E18.5 but trended toward increased expression (139%; $P = 0.06$) in P0 vs. WTL. PCR arrays demonstrated that four genes were differentially expressed in LvH WT placentas including increased expression of the calcium-binding protein calmodulin 1 (1.6-fold; $P < 0.05$). Twenty-four genes were differentially expressed in placentas of P0 vs. WTL; significant reductions were observed in expression of S100 calcium binding protein G (2-fold; $P < 0.01$), parathyroid hormone 1 receptor (1.7-fold; $P < 0.01$) and PTHrP (2-fold; $P < 0.05$), whilst serum/glucocorticoid-regulated kinase 1 (SGK1), a regulator of nutrient transporters, was increased (1.4 fold; $P < 0.05$). Tartrate resistant acid phosphatase 5 (TRAP5 encoded by Acp5) was reduced in placentas of both LvH WT and P0 vs. WTL (1.6- and 1.7-fold, respectively; $P < 0.05$). Signaling events underpinning adaptations in calcium transport are distinct between LvH placentas of WT mice and those in P0 vs. WTL. Calcium binding proteins appear important in functional adaptations in the former whilst PTHrP and SGK1 are also implicated in the latter. These data facilitate understanding of mechanisms underpinning placental calcium transport adaptation in normal and growth restricted fetuses.

Keywords: placenta, calcium, adaptation, FGR, IUGR, mouse

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *16 September 2018*
Accepted: *29 October 2018*
Published: *19 November 2018*

Citation:
Hayward CE, McIntyre KR, Sibley CP, Greenwood SL and Dilworth MR (2018) Mechanisms Underpinning Adaptations in Placental Calcium Transport in Normal Mice and Those With Fetal Growth Restriction. Front. Endocrinol. 9:671. doi: 10.3389/fendo.2018.00671

INTRODUCTION

Placental dysfunction, associated with reduced rates of nutrient uptake (1–3), is a major cause of fetal growth restriction (FGR), the failure of a fetus to reach its growth potential (4). FGR is a significant risk factor for stillbirth (5, 6). Additionally, FGR infants demonstrate increased incidence of childhood diseases such as cerebral palsy, and adulthood diseases including heart disease, stroke, diabetes and osteoporosis (7–11). The current lack of therapies for FGR (12) emphasizes the need for better understanding of how fetal development is normally achieved and how it is dysregulated in FGR.

Placental transfer of calcium increases over gestation to match fetal demand and ensure appropriate fetal skeletal mineralization (13). Poor fetal provision of calcium *in utero* has been linked with an increased risk of developing osteoporosis later in life (14). Maternofetal transfer of calcium across the placenta involves calcium moving from maternal blood into the syncytiotrophoblast (transporting epithelium of the placenta) down an electrochemical gradient through calcium permeable cation channels (e.g., TRPV6, transient receptor potential vanilloid type 6) on the maternal-facing microvillous membrane (MVM) (15–17). Once in the trophoblast cytosol calcium is buffered to avoid overly increasing the intracellular concentration, and shuttled to the fetal-facing basal membrane (BM) by calcium binding proteins such as calbindin-D_{9K} (16). Calcium is actively transported across the BM by plasma membrane calcium ATPases (PMCA) into the fetal compartment. The actions of PMCA help to maintain calcium concentrations in the fetus above those found in maternal blood. Unlike the activity of other nutrient, and especially amino acid, transport systems that are reduced in placentas of growth restricted fetuses (1–3), the activity of PMCA is increased in human FGR (18) as is the maternofetal transfer of calcium in a rodent model of FGR, the placental-specific insulin-like growth factor *2* (P0) knockout mouse (19).

Optimal fetal growth depends on adequate nutrient delivery and placental supply can be adapted to meet the metabolic needs of the developing fetus. In pregnancies with normal outcomes, adaptation of placental transport in relation to placental size appears important in both women and mice (20–22). Our previous studies of wild-type (WT) mouse litters demonstrated that maternofetal calcium transfer across the lightest placentas is adaptively up-regulated, compared to the heaviest placentas, so that all fetuses, whether with relatively lighter or heavier placentas, accrue an appropriate level of calcium relative to their size near term (22). We suggested that this increased maternofetal transfer of calcium (per gram placenta), which coincides with increased placental calbindin-D_{9K} expression at embryonic day (E)18.5, is an example of a placental adaptation that promotes fetal calcium acquisition despite a relatively small placental size. We also found a normalization of fetal calcium accretion by E18.5, following a reduction at E16.5, which may be indicative of a fetus signaling to its placenta, by as yet unknown mechanisms, to increase maternofetal transfer of calcium. The gestational timing of this adaptation in WT mice was similar to that which we previously observed in the P0 knockout mouse (19), and points to a role for fetal nutrient demand in driving this adaptation via altered expression of placental calcium binding proteins. These data showed that placental adaptations are an important feature of both normal and compromised fetal growth and help to ensure appropriate calcium acquisition relative to the size of the fetus.

Nothing is yet known regarding the underlying mechanisms that affect adaptation of placental calcium transport and in particular the fetal and/or placental signals that may be important in this process. Therefore, in this study we investigated mechanisms underlying the adaptive up-regulation of maternofetal calcium transfer. Initially, parathyroid hormone-related protein (PTHrP) was investigated as a candidate fetal signal. PTHrP is produced in a number of tissues including, but not limited to, the placenta, fetal membranes and fetal brain, liver, bone and parathyroid glands (23). In these tissues, there are multiple secretory mature peptides which have a range of different biological functions that can be elicited through endocrine, paracrine, autocrine and intracrine mechanisms [reviewed by (24)]. In women, the concentration of PTHrP in maternal serum, and PTHrP expression in amnion and choriodecidua, are both increased in late gestation in parallel with the rapid increase in fetal growth and calcium accretion (25–27). Previous studies in rodents have demonstrated the importance of PTHrP and its receptor, the PTH/PTHrP receptor, in fetal development. Deletion of these genes in mice results in neonatal death, due to skeletal dysplasia (28) or death *in utero* mid gestation due to growth restriction (29). In a spontaneously hypertensive rat model, inappropriate levels of PTHrP in the placenta, fetal plasma and amniotic fluid were associated with compromised fetal growth (30, 31). Enhancing endogenous levels of PTHrP, by the addition of a PTH/PTHrP receptor antagonist, improves fetal growth in this rat model (31). In PTHrP knockout mice, maternofetal calcium transfer and fetal calcium accretion are increased despite fetal hypocalcaemia and lack of a maternal fetal calcium gradient (32–34). In human pregnancies complicated by FGR, PTHrP expression in fetal membranes and placenta is increased in cases of preterm FGR (35), and concentrations are elevated in cord blood (18). PTHrP also stimulates PMCA activity in BM vesicles isolated from human placenta (36). Thus, we hypothesized that PTHrP is a candidate signal stimulating an increase in calcium transfer and would be elevated in: (1) placental tissue and tissues from fetuses of the lightest vs. heaviest (LvH) placentas in WT mice; and (2) in placentas of P0 fetuses compared to their WT littermates (WTL). Using calcium pathway-focused PCR arrays we also tested the hypothesis that placental mechanisms underpinning the adaptive increase in calcium transfer in WT mice and in P0 mice would be similar.

MATERIALS AND METHODS

Animals

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986 under the authority of a UK Home office project license (PPLs 40/3385 and P9755892D) and were authorized by the Animal Welfare and Ethical Review Board of the University of Manchester. The methods stated in this

study adhere to the ARRIVE guidelines (37) and comply with the animal ethical principles under which the journal operates.

Wild-type C57Bl/6J (Envigo, UK) females (10–16 weeks old) and males (12–26 weeks old) were mated and discovery of a copulation plug was used to define embryonic day (E)0.5 (term = E19.5). Mice were provided with nesting material and communally housed (with the exception of stud males that were individually housed) in individually ventilated cages under a constant 12 h light/dark cycle at 21–23°C with free access to food (Beekay Rat and Mouse diet, Bantin and Kingman) and water (Hydropac, Denver, US). Pregnant female mice were euthanized (cervical dislocation appropriate under ASPA schedule 1) and a laparotomy and hysterotomy performed. All fetuses were rapidly killed by cervical dislocation.

On E18.5 ($N = 20$ litters), pregnant WT females were euthanized and fetuses and placentas were rapidly harvested, blotted and wet weights measured. The lightest ($n = 20$) and heaviest ($n = 20$) placentas were identified in each litter. All placentas and fetuses were snap frozen and stored at −80°C. In 9/20 litters, brains ($n = 18$; 9 from the lightest placental group, 9 from the heaviest) and livers ($n = 18$; 9 from lightest, 9 from heaviest) from fetuses corresponding to the lightest and heaviest placentas were immediately dissected, snap frozen and stored at −80°C. Fetal weight histograms were constructed and a non-linear regression performed (Gaussian distribution) from which individualized fetal weight centiles were calculated as described previously (38).

Placental specific insulin-like growth factor 2 (*Igf2*) (P0) knockout mice ($N = 10$ litters), which had deletion of the U2 exon of the *Igf2* gene, were generated as previously described (39) and were a kind gift from Dr Miguel Constância and Professor Wolf Reik. C57BL/6J female mice (8–14 weeks old) and males heterozygous for the P0 deletion (10–32 weeks old) were mated and produced mixed litters of WTL fetuses and growth restricted fetuses [P0; reported birthweight 78% compared to WTL at E19 equivalent to E18.5 in the current study (40)]. Embryonic day was defined as above. At E18.5, placentas and fetuses (40 WTL; 38 P0 from 10 litters) were weighed, snap frozen and stored at −80°C. Fetal tail tips were collected from all fetuses and stored at −20°C for genotype determination.

The aim of the study, comparing lightest vs. heaviest placentas or those from WTL vs. P0 mice within a single litter, meant that randomization or blinding of the samples was not possible.

Genotyping of P0 Knockout Mice

Genotype (WTL or P0) was determined for all fetuses from P0 mice according to a previously published genotyping protocol (19, 41). In brief, genomic DNA was extracted from fetal tail tips using a DNeasy kit (Qiagen, Manchester, UK). *Igf2* $P0^{+/-}$ mutants were identified with a specific primer pair to amplify a 740 bp fragment across the 5 kb deletion (P0 dF 5′-TCCTGTACCTCCTAACTACCAC−3′ and P0 dR 5′-GAGCCAGAAGCAAACT−3′) and a primer to amplify a 495 bp fragment from the WT allele (5′-CAATCTGCTCCTGCCTG−3′). PCR conditions were as follows: 4 min denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 56°C, 1 min at 72°C; and 10 min final extension at 72°C. Samples were loaded with bromophenol blue and run on a 1.5% agarose gel. Bands were visualized using an InGenius transilluminator (Sygene Bio, Cambridge, UK).

Protein Expression

The lightest and heaviest placentas from WT mice ($N = 7$ litters) and placentas of P0 and WTL ($N = 8$ litters, 1 paired P0 and WTL placenta per litter selected at random) were homogenized and processed as described previously (19). Briefly, whole homogenates were separated, by means of centrifugation, into cytosolic fractions. Due to the small amount of starting tissue, whole homogenates of fetal tissues (brains and livers; $N = 9$ litters) were used for protein expression studies.

SDS-PAGE was performed followed by electrotransfer to Immobilon-FL PVDF membranes (Millipore UK Ltd., Watford, UK). Primary antibodies included: rabbit polyclonal antibodies for serum/glucocorticoid-regulated kinase 1 (SGK1; 1 μg/ml; ab43606; Abcam, Cambridge, UK) and calmodulin (CaM; 2 μg/ml; sc-5537; Santa Cruz Biotechnology c/o Insight Biotechnology Ltd, Wembley, UK); rabbit monoclonal antibody for tartrate-resistant acid phosphatase (TRAP; 0.9 μg/ml; ab191406; Abcam); and goat polyclonal antibody for PTHrP (1 μg/ml; N-19, sc-9680; Santa Cruz Biotechnology). β-actin (0.5 μg/ml; ab8227; Abcam) or β-tubulin (0.9 μg/ml; ab6046; Abcam) was used as a loading control; when used no difference was observed in β-actin or β-tubulin expression between groups. Negative controls were by omission of primary antibody. Immunoreactive species were detected with fluorescent-conjugated secondary antibodies (Li-COR Biosciences, Cambridge, UK) and membranes imaged using an Odyssey Sa Infrared Imaging System (Li-COR). Signal density was measured using Image Studio Lite (Li-COR). All signals were in the linear range of detection. Protein expression was compared separately between the lightest and heaviest placentas, fetal tissues from lightest and heaviest placentas, and WT and P0 samples.

RT^2 Profiler PCR Arrays

RNA was extracted from whole placentas ($N = 7$ litters: $n = 7$ lightest, $n = 7$ heaviest; $N = 6$–7 litters: $n = 6$–7 WTL, $n = 6$–7 P0, 1 paired P0 and WTL placenta per litter selected at random) using an RNeasy Mini Kit (74104; Qiagen, Manchester, UK), RNase-Free DNase set (79254; Qiagen) and measured by a Thermo Scientific NanoDrop 2000C spectrophotometer (A_{260}/A_{280} range 2.06–2.12). Any contaminating genomic DNA was removed and cDNA was synthesized from 0.5 μg RNA per sample using the RT^2 first strand kit (330401; Qiagen; genomic DNA elimination mix for 5 min at 42°C, on ice for 1 min; reverse transcription mix for 42°C for 15 min followed by 5 min at 95°C). Expression of 168 related genes, 5 reference genes and quality controls was measured in each placenta using RT^2 Profiler PCR arrays (PAMM-066Z mouse cAMP/calcium signaling pathway finder and PAMM-170Z mouse osteoporosis array; 96-well format; Qiagen) with RT^2 SYBR® Green ROX™ qPCR mastermix (330523; Qiagen) on a Stratagene MX3005P® , according to the manufacturer's instructions (10 min at 95°C, 40 cycles of 15 s at 95°C followed by 1 min at 60°C; dissociation curve 1 min

at 95°C, 30 s at 55°C, 30 s at 95°C). Data were analyzed using SA Bioscience PCR Array Data Analysis 3.5 Web Portal (http://dataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Data and Statistical Analysis

Data are presented as the lightest placenta as a percentage of the heaviest in a litter (dotted line = 100%), placentas of P0 fetuses as a percentage of WTL (dotted line = 100%), or median and [range] where the experimental N = number of litters, n = number of placentas or fetuses. The solid line on graphs represents the median value. For P0 and WTL fetal and placental weights, average fetal weights for each genotype per litter were calculated and data are shown as a mean of these average weights. Data were analyzed by Wilcoxon matched-pairs signed-rank test or Mann Whitney test. $P < 0.05$ was considered statistically significant.

PCR array data were analyzed using SA Bioscience PCR Array Data Analysis 3.5 Web Portal (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Fold change [2∧(- Delta Delta Ct)] is the normalized gene expression [2∧(- Delta Ct)] in the test sample (lightest placenta or placenta of P0 fetus) divided by the normalized gene expression [2∧(- Delta Ct)] in the control sample (heaviest placenta or placenta of WTL fetus). Fold-change values >1 indicate an up-regulation in expression, and fold-change values <1 demonstrate a down-regulation (**Supplementary Tables 1**, **2**). Fold regulation values are shown in **Tables 2**, **3**.

RESULTS

Fetal and Placental Weights

As expected, lightest placentas from WT mice demonstrated significantly reduced placental weight vs. heaviest placentas at E18.5 ($P < 0.0001$, **Table 1**). Fetal weight was significantly reduced ($P < 0.01$) and fetal weight: placental weight (F:P) ratio increased ($P < 0.0001$), in fetuses from lightest vs. heaviest WT placentas (**Table 1**). Mean fetal weight centiles were lower in the lightest compared to the heaviest placenta group (39th vs. 56th centile; $P < 0.05$) but were not considered growth restricted (normal range 10th−90th centile). Consistent with previous studies (19, 40, 41), placentas from P0 fetuses compared to WTL fetuses were lighter, fetal weight was lower and F:P ratio higher at E18.5 (all $P < 0.0001$, **Table 1**).

TABLE 1 | Placental weight, fetal weight and fetal weight:placental weight (F:P) ratio in the lightest and heaviest placental groups of wild-type (WT) mice and in P0 and wild-type littermates (WTL) at embryonic day (E) 18.5.

	Lightest	Heaviest	Lightest/ Heaviest (%)	*P*-value
Placental weight (g)	0.068 (0.052–0.080)	0.087 (0.076–0.100)	78.0 (64.0–89.0)	<0.0001
Fetal weight (g)	1.151 (0.924–1.289)	1.191 (1.018–1.441)	94.5 (79.0–108.0)	0.01
F:P ratio	16.8 (12.8–24.0)	14.2 (11.4–16.8)	119.5 (92.0–166.0)	<0.0001
	P0	**WTL**	**P0/WTL (%)**	***P*-value**
Placental weight (g)	0.065 (0.048–0.100)	0.096 (0.050–0.113)	64.0 (58.3–83.1)	<0.0001
Fetal weight (g)	0.977 (0.735–1.218)	1.179 (0.854–1.386)	80.9 (68.1–85.6)	<0.0001
F:P ratio	14.5 (10.91–18.4)	12.6 (10.0–17.1)	110.6 (100.5–146.4)	<0.0001

Data are median (range) or presented as the lightest placenta as a percentage of the heaviest in a litter (lightest/heaviest (%) column) in WT mice or as the litter average of P0 fetuses as a percentage of the litter average of their WT littermates (P0/WTL (%) column). Data are analyzed by Wilcoxon matched-pairs signed rank test (lightest vs. heaviest; P0 vs. WTL). P < 0.05 was considered statistically significant.

TABLE 2 | Results of the cAMP/Ca^{2+} signaling pathway finder and osteoporosis RT^2 profiler PCR arrays in the lightest compared to the heaviest placentas from WT mice.

Gene	Product	Fold regulation	*P*-value
Calm1	Calmodulin 1	+1.6	0.04*
Alpl	Alkaline Phosphatase	+1.2	0.04*
Acp5	Acid Phosphatase 5 Tartrate Resistant	−1.6	0.04*
Hspa5	Heat Shock 70 kDa Protein 5	−1.8	0.01*

*Fold regulation is the normalized gene expression in the lightest placentas divided by the normalized gene expression in the heaviest placentas. Fold regulation values +1 indicate an up-regulation in expression, whilst fold regulation values −1 demonstrate a down-regulation. *P < 0.05.*

PTHrP Protein Expression

There were no significant differences in PTHrP protein expression between the lightest and heaviest placentas of WT mice, or within brains and livers of those fetuses from the lightest and heaviest placentas (**Figures 1A–C,E**). There was a trend toward increased PTHrP protein expression in placental tissue of P0 vs. WTL fetuses (139%; $P = 0.06$; **Figures 1D–E**). The PTHrP antibody used for these studies was discontinued during the timecourse of the project and so we were unable to assess PTHrP expression in fetal brains and livers of fetuses in P0 vs. WTL mice. Attempts to use different antibodies targeted to PTHrP failed to show reproducible amplification of signal.

RT^2 Profiler PCR Arrays

PCR arrays demonstrated significant changes in the expression of four genes (**Table 2**); an increase in calmodulin 1 (*Calm1*; $P < 0.05$) and alkaline phosphatase (*Alpl*; $P < 0.05$) expression, and a decreased expression of tartrate resistant acid phosphatase 5 (*Acp5*; $P < 0.05$) and heat shock protein 5 (*Hspa5*; $P < 0.05$) in the lightest vs. heaviest WT placentas ($N = 6$). Placental protein expression of calmodulin (CaM; 107%; **Figure 2A**) and tartrate resistant acid phosphatase 5 (TRAP; 89%; **Figure 2B**) measured by Western blot was not different between lightest and heaviest placenta groups.

Twenty-four genes were differentially expressed in placentas from P0 vs. WTL fetuses in the same litter as shown in **Table 3**. Of note, significantly reduced expression was observed in the genes encoding calbindin-D_{9K}, S100 calcium binding protein G (*S100g*; −1.9-fold, $P < 0.01$), PTHrP (*Pthlh*; −2-fold, $P <$

TABLE 3 | Results of the cAMP/Ca^{2+} signaling pathway finder and osteoporosis RT^2 profiler PCR arrays between placentas from P0 compared to wild-type littermate (WTL) fetuses.

Gene	Product	Fold regulation	*P*-value
Ar	Androgen Receptor	+1.7	0.04*
Esr1	Estrogen Receptor	+1.5	0.02*
Sgk1	Serum/Glucocorticoid-Regulated Kinase 1	+1.4	0.03*
Eno2	Enolase 2, γ Neuronal	+1.4	0.03*
Tnfrsf11b	Tumor Necrosis Factor Receptor Superfamily, 11b	+1.4	0.001**
Tgfb3	Transforming Growth Factor, β3	−1.2	0.04*
Rb1	Retinoblastoma 1	−1.3	0.02*
Pck2	Phosphoenolpyruvate Carboxykinase 2 (Mitochondrial)	−1.3	0.04*
Junb	Jun-B Oncogene	−1.3	0.04*
Fosb	FBJ Osteosarcoma Oncogene B	−1.4	0.02*
Crem	cAMP Responsive Element Modulator	−1.4	0.03*
Per1	Period Homolog 1 (Drosophila)	−1.4	0.04*
Nos2	Nitric Oxide Synthase 2, Inducible	−1.4	0.006**
Brca1	Breast Cancer 1	−1.5	0.01*
Acp5	Acid Phosphatase 5, Tartrate Resistant	−1.7	0.02*
Cyp17a1	Cytochrome P450 Family 17, a1	−1.7	0.03*
Ltbp2	Latent Transforming Growth Factor β Binding Protein 2	−1.8	0.03*
Gem	GTP Binding Protein (over-expressed in skeletal muscle)	−1.8	0.04*
S100g	S100 Calcium Binding Protein G	−1.9	0.004**
Dkk1	Dickkopf Homolog 1	−1.9	0.005**
Wnt10b	Wingless Related MMTV Integration Site 10b	−1.9	0.04*
Car2	Carbonic Anhydrase 2	−2.0	0.009**
Pthrp	Parathyroid Hormone-Related Protein	−2.0	0.02*
Ncam1	Neural Cell Adhesion Molecule 1	−2.0	0.002**

Fold regulation is the normalized gene expression in placentas of P0 fetuses divided by the normalized gene expression in placentas of WTL fetuses. Fold regulation values +1 indicate an up-regulation in expression, whilst fold regulation values −1 demonstrate a down-regulation. $^{*}P < 0.05$; $^{**}P < 0.01$.

0.05) and parathyroid hormone 1 receptor (*Pth1r*; −1.7-fold, $P < 0.01$). Expression of serum/glucocorticoid-regulated kinase 1 (*Sgk1*; 1.4-fold, $P < 0.05$), a kinase involved in the regulation of a range of membrane transporters, ion channels and transcription factors as well as cell survival (42–46) was increased. There was no difference in serum/glucocorticoid-regulated kinase 1 (SGK1; 88%; **Figure 3**) protein expression between placentas of P0 and WT mice.

There was only one similar change in gene expression between the two study groups; tartrate resistant acid phosphatase 5 (*Acp5*, −1.6-and −1.7-fold, respectively) was reduced to the same extent in the lightest placentas of WT mice and placentas of P0 fetuses (**Tables 2**, **3**). Whilst TRAP protein expression was no different between lightest and heaviest WT placentas, gene expression of TRAP was increased in placentas of P0 compared to WT littermates (TRAP; 144%; **Figure 2C**).

DISCUSSION

We have previously observed similar adaptive increases in maternofetal calcium transport in small placentas from WT mice, and in small pathological placentas of the P0 knockout mouse model of FGR, with accompanying changes in calbindin-D_{9K} expression (19, 22). Here we demonstrate that the underlying mechanisms of these adaptations in the two models appear to be distinct. Contrary to our hypothesis, expression of PTHrP was not different between lightest compared with heaviest (LvH) WT placentas, but there was a trend toward increased expression in P0 vs. WTL placentas; PTHrP has been shown to influence placental calcium transport in most (32, 33, 36), but not all studies (47). As such, any change in PTHrP expression in placentas near term may be important in the increased maternofetal calcium transfer observed in P0 vs. WTL.

As shown by our previous studies, calbindin-D_{9K} is implicated as a mediator of placental adaptation in calcium transfer both in WT mice (22) and in the P0 mouse (19). However, the lack of change in placental calcium transfer in the calbindin-D_{9K} knockout mouse indicates that other candidates, including calbindin-D_{28K}, TRPV5/6 and the sodium-calcium exchanger, might also be involved (48, 49). Thus, we adopted a holistic approach to compare the expression of genes related to calcium transfer and signaling in WT mice and P0 mice. We speculated that these experiments would identify similar calcium-specific pathways altered in the lightest and/or P0 placentas and provide insight into potential regulators of the observed placental adaptation. The number of genes showing altered expression was limited in lightest vs. heaviest WT placentas but mRNA expression of calmodulin-1 was increased in the lightest placentas at E18.5. Whilst this may act as a further indicator of the

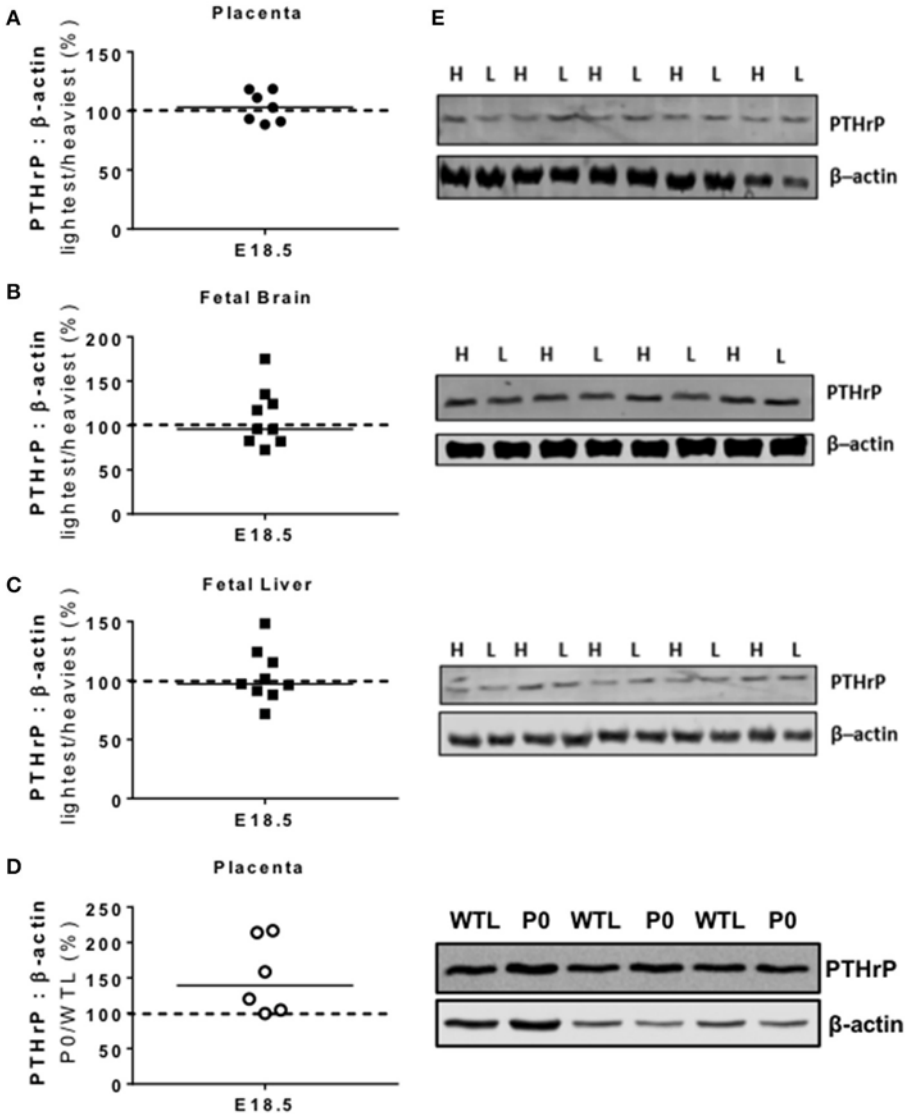

FIGURE 1 | Placental and fetal protein expression of parathyroid hormone-related protein (PTHrP). PTHrP protein expression was not significantly different in paired lightest (L) and heaviest (H) placentas from the same litter **(A)** or in the brains **(B)** and livers **(C)** of the fetuses from these placentas. **(D)** There was a trend for higher PTHrP protein expression in placentas of paired placental-specific insulin-like growth factor 2 knockout (P0) and wild-type (WTL) fetuses ($P = 0.06$) from the same litter. **(E)** Representative Western blots of PTHrP (26 kDa) with the corresponding loading control (β-actin; 42 kDa). Black line = median; dotted line 100% = H or WTL placenta.

importance of calcium binding proteins in the previously observed adaptation, this altered expression was not mirrored at the protein level; the importance of the change in gene expression therefore requires further elucidation.

In contrast to the lightest and heaviest placentas in WT mice, multiple genes were differentially expressed in placentas of P0 vs. WT littermates. This is perhaps unsurprising given that P0 mice represent a model of fetal growth restriction whereas lightest vs. heaviest placentas represent extremes of placental weight in a "normal" WT population. Despite the increased maternofetal calcium transport at E18.5, expression of placental calcium-related genes was generally reduced in P0 vs. WTL. Significant reductions were observed in the expression of *S100g* (encoding calbindin-D_{9K}), *Pth1r* and *Pthrp*, whilst *Sgk1*, a regulator of epithelial ion transport and cell survival, was up-regulated. SGK1 is a downstream effector of the PI3K/AKT signaling pathway, and in support of the observations here, this pathway is dysregulated in the placentas of P0 knockout mice in late gestation (50). The trend for reduced gene expression near to term could be the result of timing in gestation, i.e., gene expression increased earlier in gestation to promote changes in placental nutrient transport might be downregulated nearer to term having already resulted in increased transcription of the target protein, as previously observed for calbindin-D_{9K} in

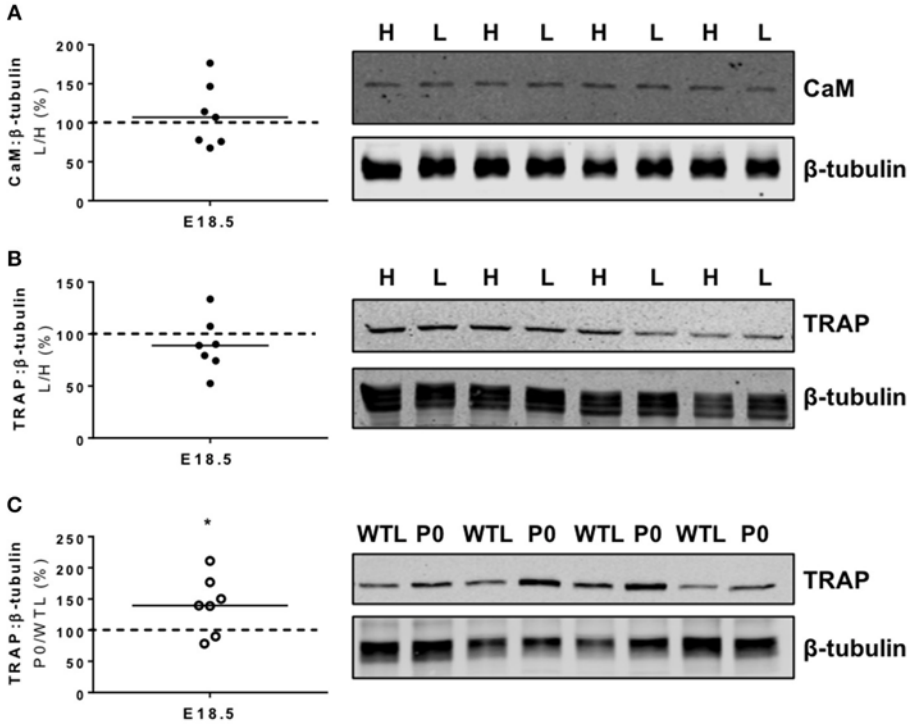

FIGURE 2 | Placental protein expression of calmodulin (CaM) and tartrate resistant acid phosphatase 5 (TRAP). **(A)** CaM and **(B)** TRAP protein expression was not significantly different in paired lightest (L) and heaviest (H) WT placentas from the same litter. **(C)** TRAP protein expression was increased in placentas of placental-specific insulin-like growth factor 2 knockout (P0) compared to wild-type littermates (WTL) from the same litter (*$P < 0.05$; Wilcoxon signed rank test). Black line = median; dotted line 100% = heaviest WT placenta **(A,B)** or WTL placenta **(C)**. Detected band sizes from representative western blots were as follows; CaM (17 KDa), TRAP (42 kDa) and ß-tubulin loading control (50 kDa).

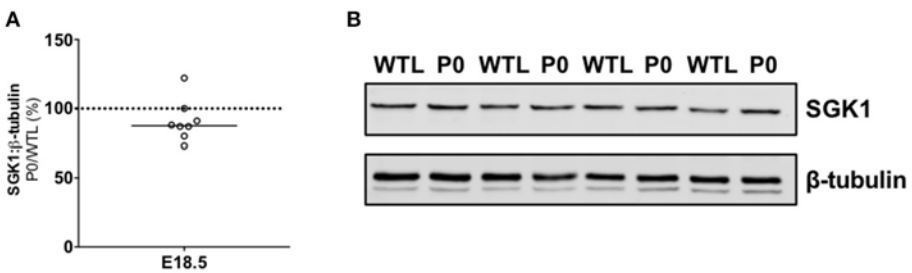

FIGURE 3 | Placental protein expression of serum/glucocorticoid-regulated kinase 1 (SGK1). **(A)** SGK1 protein expression was not significantly different in paired placentas of placental-specific insulin-like growth factor 2 knockout (P0) and wild-type (WTL) fetuses from the same litter. **(B)** Representative Western blots of SGK1 (49 kDa) with the corresponding loading control (β-tubulin; 50 kDa). Black line = median; dotted line 100% = WTL placenta.

lightest vs. heaviest WT placentas (22). The choice of E18.5 for these studies reflected the timepoint at which the adaptation (increased placental calcium transport) was previously observed (19, 22) but analyses earlier in gestation would offer further insight into the timing of these changes. With regards to the trend for reduced gene expression near term in P0 vs. WTL, altered gene expression may not be the driving force in these placentas; instead post-translational processing of binding proteins and/or receptors (e.g., TRPV6) may underlie the adaptive changes in calcium transfer. Mechanisms will need to be explored further in future experiments. Whilst the discrepancy in *S100g* gene and calbindin-D_{9K} protein expression at E18.5 will need elucidating, calbindin-D_{9K} does appear to be important in the previously reported changes in placental calcium transport in mouse models of FGR (19, 22).

Increased *Sgk1* expression in P0 vs. WT warrants further investigation to assess whether SGK1, and its activated phosphorylated isoform, play an important role in these adaptive responses by the placenta. SGK1 influences intracellular calcium by up-regulating store operated calcium entry (SOCE),

increasing calcium release-activated calcium channel (CRAC) current, and increasing the activity of TRPV5 and 6 (46). Expression and activation of SGK1 is enhanced by higher levels of cytosolic calcium thus SGK1 has been suggested as an amplifier of calcium entry; influx of extracellular calcium through SOCE combined with activation of calcium/calmodulin protein kinase signaling up-regulates levels and activity of SGK1 (42–44). Activation of SGK1 also occurs through other mechanisms, including through the phosphatidylinositol-3-kinase pathway that when stimulated by growth factors activates the mechanistic target of rapamycin complex 2 triggering the phosphorylation of 3-phosphoinositide-dependent kinase PDK1 and subsequent phosphorylation of SGK1 (45, 51). Identifying extracellular regulators stimulating SGK1 intracellular activity may provide potential candidate signals initiating placental adaptations.

Placental *Acp5* gene expression was lower in the lightest compared to the heaviest placentas and in P0 fetuses compared to their WTLs. In contrast, TRAP5 protein expression (encoded by *Acp5*) was not different between the lightest and heaviest placentas, and significantly higher in placentas of P0 compared to WTLs. Previous studies in animal models indicate *Acp5* has an essential role in modeling, remodeling and mineralization of developing bone and cartilage (52), as well as participating in iron transfer from mother to fetus (53). Hansson et al. (54) demonstrated increased placental *Acp5* gene expression in pre-eclampsia compared to normal pregnancy and suggested that this increase might be a compensatory mechanism for poor placentation to prevent fetal malnutrition (54). The elevated expression of TRAP5 protein in placentas of P0 vs. WTL fetuses, which may be as a result of increased gene expression earlier in gestation leading to increased protein translation, supports a regulatory role for TRAP5 in this mouse model of FGR; the added complexity of the opposing direction of change in gene and protein expression, suggest post-translational modification is very important.

For all of the studies described herein, there was an unequal distribution of fetal sex when considering the placental samples analyzed. For the studies comparing lightest vs. heaviest placentas in WT mice, there was a bias toward females having the lightest placentas and males having the heaviest placentas. Whilst we have previously reported that adaptive changes in maternofetal calcium transport do not appear to be influenced by fetal sex (22), future studies investigating the mechanisms underpinning these adaptations should also consider sex-dependent effects. Likewise, fetal sex should be taken into account when assessing mechanisms underpinning placental adaptations in P0 vs. WTL mice.

In summary this study has shown differences in the mechanisms underlying adaptations in placental calcium transport in normal pregnancy vs. that affected by growth restriction. Our data suggest that calcium binding proteins in normal mouse pregnancy, and PTHrP and *Acp5*/TRAP in FGR (P0) pregnancy, are candidate adaptation regulatory proteins worthy of further investigation.

AUTHOR CONTRIBUTIONS

KM performed research. SG and CS contributed to the conception and design of the work. CH and MD contributed to the conception and design of the work and performed research. All authors were involved in drafting the paper.

FUNDING

This study was supported by a Medical Research Council Career Development Fellowship award (Grant number MR/K024442/1), and awarded to MD.

ACKNOWLEDGEMENTS

We would like to thank the staff of the Biological Services Facility at the University of Manchester for their assistance with this project. Infrastructure support was provided by the National Institute for Health Research Manchester Biomedical Research Centre and Tommy's the Baby Charity.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00671/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 08 January 2019
doi: 10.3389/fphys.2018.01884

The Possible Role of Placental Morphometry in the Detection of Fetal Growth Restriction

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Fetal growth restriction (FGR) is often the result of placental insufficiency and is characterized by insufficient transplacental transport of nutrients and oxygen. The main underlying entities of placental insufficiency, the pathophysiologic mechanism, can broadly be divided into impairments in blood flow and exchange capacity over the syncytiovascular membranes of the fetal placenta villi. Fetal growth restriction is not synonymous with small for gestational age and techniques to distinguish between both are needed. Placental insufficiency has significant associations with adverse pregnancy outcomes (perinatal mortality and morbidity). Even in apparently healthy survivors, altered fetal programming may lead to long-term neurodevelopmental and metabolic effects. Although the concept of fetal growth restriction is well appreciated in contemporary obstetrics, the appropriate detection of FGR remains an issue in clinical practice. Several approaches have aimed to improve detection, e.g., uniform definition of FGR, use of Doppler ultrasound profiles and use of growth trajectories by ultrasound fetal biometry. However, the role of placental morphometry (placental dimensions/shape and weight) deserves further exploration. This review article covers the clinical relevance of placental morphometry during pregnancy and at birth to help recognize fetuses who are growth restricted. The assessment has wide intra- and interindividual variability with various consequences. Previous studies have shown that a small placental surface area and low placental weight are associated with a slower growth of the fetus. Parameters such as placental surface area, placental volume and placental weight in relation to birth weight can help to identify FGR. In the future, a model including sophisticated antenatal placental morphometry may prove to be a clinically useful method for screening or diagnosing growth restricted fetuses, in order to provide optimal monitoring.

Keywords: FGR, IUGR, SGA, fetal growth restriction, intra uterine growth restriction, small for gestational age, placenta morphometry, birth weight

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *26 September 2018*
Accepted: *12 December 2018*
Published: *08 January 2019*

Citation:
Salavati N, Smies M, Ganzevoort W, Charles AK, Erwich JJ, Plösch T and Gordijn SJ (2019) The Possible Role of Placental Morphometry in the Detection of Fetal Growth Restriction. Front. Physiol. 9:1884. doi: 10.3389/fphys.2018.01884

BACKGROUND

The diagnosis of fetal growth restriction (FGR) has for long mainly be based on birth weight below a reference cut-off, most commonly the 10th percentile (p10) (Beune et al., 2018). Birth weight (BW) or estimated fetal weight (EFW) below p10 indicates that the BW or EFW is within the lowest 10% of BW compared to the reference population. This is in essence not FGR but small

for gestational age (SGA). There are some important diagnostic issues with this misnomer. First, about 75% of fetuses who are SGA (and therefore many who are FGR) remain unrecognized until they are born and the diagnosis is made on the baby scale, postnatally (Monier et al., 2017; Beune et al., 2018), meaning some are severely compromised, exposed to potential long term sequelae, or even stillborn. Second, fetuses who are too small according to the intra uterine reference chart may be physiologically small and appropriate grown according to their individual growth potential (based upon their genetic and epigenetic inheritance at conception), and therefore not at risk from diseases related to FGR, but are exposed to unnecessary investigations for FGR. Third, many cases of growth restriction remain unacknowledged, when a baby or fetus is too small according to its *individual* growth potential, but not necessarily too small in the *population* based reference chart. Thus FGR overlaps with, but is not synonymous to, SGA (Zhang et al., 2010) ("SGA-FGR confusion"), as two overlapping distribution curves. It is self-evident that the incidence of growth restricted fetuses increases as EFW or BW percentiles decreases (Vasak et al., 2015). Yet, there is not a single cut-off above which all babies have grown appropriately, or below which none have grown appropriately for their individual biological growth potential. If SGA is used as the proxy for FGR in clinical practice, healthy SGA fetuses and neonates without FGR are prone to unnecessary monitoring intervention strategies and FGR fetuses and neonates who are FGR but not SGA remain unrecognized ("masked" FGR). Furthermore, if SGA is used as proxy for FGR in research, the study population is diluted by healthy small fetuses and newborns, hampering adequate association studies. It is estimated that in the SGA group, 60% were growth restricted, and 40% were constitutionally small (**Figure 1**) (Figueras and Gratacos, 2017). In this study, "constitutionally small" was defined as fetuses with moderately low BW (>3rd percentile) and normal placental function on both the fetal (normal cerebroplacental ratio) and maternal (normal uterine Doppler) sides. Severe growth restriction or evidence of placental dysfunction was defined as "growth restricted" (Figueras and Gratacos, 2017).

This study implies the relevance of appropriate, possibly easy to obtain, and cheap diagnostic tools to detect only those fetuses who are growth restricted because these are the fetuses who have an increased risk of adverse short- and long-term outcomes when not delivered in time (Jaddoe et al., 2014; Meher et al., 2015; Miller et al., 2016).

The causes of FGR can be divided into pre-utero-placental (e.g., maternal anemia, hypoxia, malnourishment), utero-placental (e.g., poor implantation, pre-eclampsia) and fetal conditions (e.g., fetal infection, some maldevelopments), and conditions like twin to twin transfusion. The utero-placental group appears the largest (ACOG Practice Bulletin no. 134: Fetal Growth Restriction, 2013), and the main focus to date has been on histopathological changes such as maternal malperfusion, villitis and more recently terminal villous hypoplasia. However, recently more focus has been made on examining the role of the gross examination of the placenta, with weight, shape, cord insertion. With better identification of the factors that are associated or causative for FGR, the baby who may or may not be small can still be identified as at risk for sequelae of FGR, based upon the severity of the changes. The issue is complex as the relationship is not straightforward, and several opposing forces are occurring. The placenta is not inert and does not grow purely as its genes dictate, but it appears to respond to the demands of the fetus and also the supply from the mother, appearing to adapt and compensate. It does this on a local level controlling blood flow through the stem villi with the arterial muscle, and globally, causing the increased placental resistance that in turn is identified by the Doppler studies. Furthermore, the fetus does the same with its redistribution of the blood, to the brain, at the cost of the liver glycogen and fatty tissue stores. There may also be a tradeoff on how much of the overall nutrient going to the conceptus is used for the placenta (to maximize uptake) or to the fetus, but usually the birth weight to placenta weight-ratio (BWP-ratio) increases in conditions with FGR. In addition, there are also well established differences between male and female fetuses.

Ideally for every fetus, all the relevant factors are assessed to give a rational approach to answer the question whether the fetus has reached its full growth percentile, based on the assessment of significant evidence of less than optimal maternal factors, uteroplacental factors including gross and histopathological placental examination, and fetal factors.

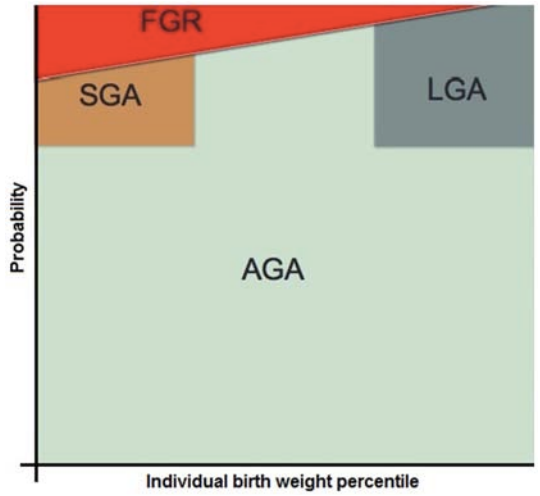

FIGURE 1 | Schematic representation of the possible distribution of FGR within the total population consisting of SGA, AGA, and LGA fetuses at a certain gestational age. Another gestational age-period or population will most likely have a different distribution. FGR, fetal growth restriction; SGA, small for gestational age; AGA, appropriate for gestational age; LGA, large for gestational age [reproduced with permission from (Gordijn et al., 2018)].

Abbreviations: AGA, appropriate for gestational age; BW, birth weight; CPR, cerebroplacental ratio; EFW, estimated fetal weight; FGR, fetal growth restriction; GA, gestational age; GV, growth velocity; HC, head circumference; LGA, large for gestational age; MRI, Magnetic Resonance Imaging; PAPP-A, pregnancy-associated plasma protein A; PI, pulsatility index; PIGF, placenta growth factor; PT, placental thickness; PV, placental volume; PW, placental weight; PQ, placenta quotient (=PV/gestational age); sFLIT1, soluble fms-like tyrosine kinase 1; SGA, small for gestational age; UmA, umbilical artery; UtA, uterine artery.

In this literature review we focus of the gross examination of the placenta and we aim to give an overview on the possible use of *placental morphometry* in recognizing fetuses and neonates with growth restriction, independent of their weight.

Diagnosis of Fetal Growth Restriction

The most common pathophysiologic mechanism of FGR is placental insufficiency, with multiple underlying maternal, fetal and placental causes, resulting in insufficient nutrition and oxygen supply to the fetus (ACOG Practice Bulletin no. 134: Fetal Growth Restriction, 2013). As mentioned above, the diagnostic process is complicated by the "SGA-FGR confusion." Placental insufficiency, placing fetuses at increased risk of hypoxia and malnourishment related morbidity, as well as stillbirth, is not restricted to those fetuses who are growth restricted and small, but also to those within normal weight ranges. The "masked" FGR fetuses in the subgroup of appropriate for gestational age (AGA) or even large for gestational age (LGA) fetuses, also experience placental insufficiency [assessed by umbilical artery pulsatility index (PI), middle cerebral artery PI, cerebroplacental ratio] (Morales Roselló et al., 2012). They show a slower growth trajectory during pregnancy, and are prone to the same risks. In addition, these fetuses have a further doubling of stillbirth risk compared to those fetuses with detected FGR, because no interventions to modify that risk are installed (Lindqvist and Molin, 2005; Gardosi et al., 2013). Clinically, these fetuses can only be recognized with sequential ultrasound measurements that show a decline in weight centiles, which is not routinely applied in general midwifery and obstetric practice. FGR, particularly early onset, is associated with histopathological changes through later onset may be histologically unremarkable (Mifsud and Sebire, 2014).

The major challenge of FGR is the diagnostic standard. In 2016 an international Delphi procedure among 56 experts on FGR was established to come to a consensus definition for both early ($<$32 weeks of gestation) and late FGR ($\geq$32 weeks of gestation) (Gordijn et al., 2016). In this definition not only size parameters of the fetus but also parameters of placental function, either alone or in combination, are included and have been used widely since publication. The clinical applicability of these definitions in predicting adverse outcomes is yet to be assessed.

The Role of Placental Size in Fetal Growth

Normal growth of the fetus is mainly dependent on normal placental function, with normal placental morphometry (size and shape) and normal structure. Impairments in placental development, including reduced placental size, or altered placental nutrient transport capability contribute to placental dysfunction (Zhang et al., 2015). Placental dysfunction attributable to structural fetal or genetic fetal defects share similar pathophysiologic pathways but are characterized by a different set of pathophysiologic features and are not included in this review. These factors contributing to placental dysfunction, as well as changes in the placental transport system, result in FGR.

To illustrate, it is known that transporter activity of system A amino acid uptake is reduced in placentas from FGR fetuses with abnormal umbilical artery Dopplers, and that it is also related to the severity of FGR (Glazier et al., 1997). The capability of the placenta to maintain sufficient nutrient supply is commonly described as "placental efficiency" and is described to be reflected by BWPW-ratio (Wilson and Ford, 2001). The increased risk of stillbirth may reflect less "placental reserve" with a high BWPW-ratio, where the fetus is running higher risk of stillbirth and yet maximizing its albeit constrained weight and growth. In animal studies, positive correlations were found between BWPW-ratio and placental uptake of nutrient transport system A amino acid uptake, indicating that nutrient transfer per gram placenta must have increased compared to low or normal BWPW-ratio (Hayward et al., 2016). However, in human studies these effects are less conclusive regarding the system A transporter. A low BWPW-ratio describes fetuses with a relatively large placenta (higher placental weight) compared to the birth weight, whereby the nutrient transfer is reduced per gram placenta.

Antenatal Measurement of Placental Function

Prenatal screening for FGR in general obstetrical populations involves identifying risk factors for impaired fetal growth. When the fetus is identified to be at risk for FGR, sequential assessment of fetal size, either by anatomical reference points or by sequential ultrasound is executed. Actual fetal size reflects past placental function until that point in time, whereby "real time" placental function can be assessed *in vivo* by measurement of vascular resistance Doppler flows in the mother (uterine artery) or in the fetus (umbilical artery and middle cerebral artery) (Alfirevic et al., 2010). Doppler flow measurements enable the non-invasive detection of signs of placental insufficiency and fetal hemodynamic changes that occur during oxygen deprivation. During the course of normal, healthy, pregnancies, umbilical artery resistance decreases gradually throughout gestation, and increases with placental insufficiency (Unterscheider et al., 2013). Ghosh et al. suggested in their study on pregnancies complicated by suspected FGR fetuses that abnormal Doppler patterns of the uterine arteries could identify a fetus at increased risk, even in the presence of normal umbilical artery Doppler flow (Ghosh and Gudmundsson, 2009). Regarding fetal biometry, abdominal circumference is smaller in FGR fetuses due to depletion of abdominal adipose tissue as well as smaller liver size, because of reduced glycogen storage. As solitary parameter in the detection of FGR, measurement of the abdominal circumference is the most sensitive (Nardozza et al., 2017). In case of placental insufficiency and decreased oxygen and nutrients supply, the fetus redistributes the blood to the brain, at the costs of the liver glycogen and fatty issue stores, resulting in normal fetal brain growth and decline of growth of the abdominal circumference.

Measurement of Serum Biomarkers

Biochemical biomarkers that reflect placental function and adverse pregnancy outcomes, including FGR, are increasingly subject of research. A systematic review conducted in 2013, assessed 53 studies investigating biomarkers that could potentially have a role in screening for FGR. They concluded that none of the 37 different biomarkers were sufficiently accurate

to function as a predictor of FGR (Conde-Agudelo et al., 2013). However, different definitions of FGR were used in the evaluated studies in which the vast majority; 47 of the 53 studies, used SGA as a proxy for FGR. Gaccioli and colleagues, investigated whether an EFW below the 10th percentile in combinations with an elevated sFLIT1: PIGF ratio (at 36 weeks of gestation) was predictive for adverse pregnancy outcomes. They showed that this combination was strongly predictive for delivering a SGA infant (birth weight < 10th centile) plus perinatal morbidity and/or preeclampsia (Gaccioli et al., 2018a). In a subsequent study, elevated FLIT1:PIGF ratio was combined with low abdominal circumference growth velocity (ACGV), and a composite measure generated by, the earlier mentioned, Delphi procedure (Gordijn et al., 2016), described as indicators of FGR (Gaccioli et al., 2018b). They found that at a gestational age of 28 as well as 36 weeks, the positive predictive value of ultrasonic screening for the delivery of a SGA infant with complications was doubled when it was combined with biochemical markers compared to the ultrasonic screening method alone. The relation with placental morphometry has not been examined in these studies.

CLINICAL RELEVANCE OF ANTENATAL AND POSTNATAL ASSESSMENT OF PLACENTAL MORPHOMETRY

In current clinical practice, placental morphometry is only routinely investigated and described at pathological examination, after delivery. In general, placentas are only routinely investigated in case of some adverse pregnancy outcomes. However, examination of placental morphometry, both in the antenatal and postnatal phase, can possibly disclose information for the detection of fetal growth restriction.

Antenatal Assessment of Placental Morphometry

Evaluation of the placenta during pregnancy is usually only performed to assess the location of the placenta or to diagnose placental adhesion disorders (e.g., *placenta praevia*, *placenta increta*, *placenta bilobata*). Antenatal assessment of placental morphometry, alone or in relation to fetal size, is not routinely performed but may improve the identification of fetuses at risk for adverse outcomes caused by placental insufficiency. The theoretical advantage of antenatal assessment compared to postnatal assessment of placenta morphometry is obvious; relevant information from antenatal assessment does not only apply to the newborn or future pregnancies, but could also be of vital importance in the current pregnancy. It can allow the clinician to tailor monitoring- and intervention strategies to reduce the risk of adverse outcomes.

Unfortunately, literature regarding the clinical relevance of antenatal assessment of placental morphometry is still scarce. In this paragraph, we will summarize the existing literature on placental morphometry assessment during pregnancy with both ultrasound and MRI.

Antenatal Assessment of Placental Morphometry With Ultrasound

Measurements of placental diameter and thickness, using two-dimensional ultrasound, have been used as indicator of high-risk pregnancies and correlates with birth weight (Afrakhteh et al., 2013). Several studies have investigated these ultrasound measures in relation to SGA (birth weight < p10), and showed that placental diameter and thickness are lower in SGA fetuses (Habib, 2002; Afrakhteh et al., 2013; Mathai et al., 2013; Schwartz et al., 2014). In addition, Schwartz and colleagues had the aim to combine early, direct ultrasound assessment of the placenta with other markers of placental development, such as mean of the uterine artery Doppler PI, to identify pregnancies delivering SGA infants (Schwartz et al., 2014). Placental volume, placental quotient (PQ = placental volume/gestational age), and mean placental diameter were significantly smaller in fetuses in the SGA group, compared to the AGA group. This indicates that a smaller placental mass is associated with SGA (Heinonen et al., 2001; Chisholm and Folkins, 2016).

On the other hand, the placental morphology index [defined by mean placental diameter divided by placental quotient (PQ)] was significantly higher in the SGA group, demonstrating a closer association between slower fetal growth and a relatively wide and flat placenta, rather than a relatively thick placenta (Schwartz et al., 2014).

Studies that used FGR as outcome variable (**Table 1**) showed that abnormal placental shape (placental thickness > 4 cm or >50% of placental length) were predictive for experiencing FGR (Viero et al., 2004; Toal et al., 2007, 2008). In addition, Proctor et al. showed that FGR was associated with small placental size (linear placental length <10 cm), in a group of women with low first trimester PAPP-A (≤0.30 multiples of median) (Proctor et al., 2009).

In order to assess placental morphometry during pregnancy with ultrasound, sonographic reliability of placental measurements has to be adequate. In this regard, a couple of limitations have to be addressed. First, there are no existing, *in vivo*, ultrasound reference charts of normal placental size. Although Higgins et al. described that the estimated placental biometry and volume during pregnancy are correlated with their measurements at postnatal assessment, they are not equal (Higgins et al., 2016). *In vivo* measurements, performed within 7 days before delivery, of placental length and width, and 3D placental volume measurements were smaller compared to *ex vivo* measurements (Higgins et al., 2016). Placental depth and 2D placental volume measurements were found to be larger compared to their *ex vivo* correlates. These differences are probably caused by the collapse of intervillous space due to loss of maternal blood flow after birth and less stretching of the placenta due to the loss of intrauterine pressure from amniotic fluid and the baby volumes after birth. Azpurua et al. described that placental weight could be accurately predicted by 2D ultrasound with volumetric calculation (Azpurua et al., 2010). Second, intra- and inter-observer variability play a much bigger role with *in-vivo* sonographic measurements than *ex-vivo*, real life measurements (Higgins et al., 2016). Higgins et al. investigated the intra- and inter-observer variability between observations

TABLE 1 | Literature overview of antenatal placental morphometry assessment with ultrasound in relation to FGR (markers).

Author, year, country	Study type and population	FGR definition	Determinant(s)	Outcome	Results
Viero et al., 2004, Canada	Prospective 60 SP with ARED flow velocities in UmA.	EFW <p10 and - Elevated HC/AC - Amniotic fluid index (AFI) <10 cm	PL, PT, cord insertion Small/thick placenta: max PT > 4 cm, in absence of uterine contraction OR >50% of PL	Pregnancies with ARED in UmA.	Ultrasound was accurate in identifying lateral or marginal cords (sensitivity 86%, PPV 71%; $p < 0.0001$) Ultrasound examination of the placenta and its maternal blood supply may contribute to the perinatal management of pregnancies with high risk of perinatal morbidity/mortality.
Toal et al., 2007, Canada	Prospective 212 high risk* SP	EFW <p10 delivered at <34 wks and - Ultrasound examinations demonstrating reduced fetal growth - AFI <10 cm - ARED in UmA	Abnormal placental shape: PT > 4 cm or >50% of PL. Abnormal placental cord insertion. Placental texture. GA: 18–23 wks	FGR	The odds of the development of FGR were sign. less in women with all normal test results. Combining those women with two ($n = 21$) of three ($n = 3$) abnormal test results together predicted 14 of 19 severe FGR.
Toal et al., 2008, Canada	Prospective 60 high risk* SP with abnormal UtA Doppler flow at GA 19–23 weeks	EFW <p10 delivered at <34 wks and -Ultrasound examinations demonstrating reduced fetal growth - AFI < 10 cm - Abnormal UmA Doppler waveforms	Abnormal placental shape (PT/PL ratio of >0.5 or PT of >4 cm)	FGR	Women with abnormal placental shape had higher odds of FGR (odds, 4.7; 95% CI, [1.6–14.1]). Combined abnormal UtA Doppler flow and placental dysmorphologic condition before fetal viability identifies a subset of women who are at risk for adverse outcomes.
Proctor et al., 2009, Canada	Prospective 90 SP with first trimester PAPP-A ≤ 0.30 multiples of median.	EFW <p10 with UmA PI >p95 or ARED in UmA, and - Serial fetal biometry demonstrating growth failure. - AFI < 5 cm	Placental size (linear placental length). GA: 18–24 wks	FGR, preterm delivery before 32 weeks, stillbirth	FGR was sign. associated with small placental size (linear placental length <10 cm), in group of women with low PAPP-A.

** defined as "significant medical and/or obstetric risk factors for hypertensive disease/placental insufficiency."*

AC, abdominal circumference; AFI, amniotic fluid index; ARED, absent or reversed end diastolic; EFW, estimated fetal weight; FGR, fetal growth restriction; GA, gestational age; HC, head circumference; PAPP-A, pregnancy-associated plasma protein A; PL, placental length; PPV, positive predictive value; PT, placental thickness; SP, singleton pregnancies; UmA, umbilical artery; UtA, uterine artery; wks, weeks.

for placental measurements length, width, depth and volume performed by 2D ultrasound. The variability in measurements (intra- and inter-) was suboptimal with no intraclass correlation coefficient >0.75 (Higgins et al., 2016). More recently, a new technique was established for estimating placental volume from 3D ultrasound scans through an semi-automated technique (Looney et al., 2018). In this study, placental volume of 2,393 pregnancies was assessed by three operators on the one hand, and this semi-automated tool on the other hand. The clinical utility of placental volume was tested by looking at prediction of SGA at term. Results showed good similarity between the operators and the tool, and almost identical clinical results for the prediction of SGA (Looney et al., 2018).

Antenatal Assessment of Placental Morphometry With Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is an established, safe method of imaging during second and third trimester of pregnancy, but currently mainly used for fetal imaging (Wang et al., 2012a,b; Bulas and Egloff, 2013). The advantages of MRI compared to ultrasound, are the more accurate measurements of anatomical volume and the higher soft tissue contrast, and thus it has specific strengths in detecting abnormal placental morphometry. Furthermore, it has a larger field of view and, other than ultrasound, it is not dependent on its ability to penetrate tissue.

Reference values of placental volume by MRI measurements throughout gestation of healthy pregnancies, although in relatively small sample size, have been studied and are available now (Duncan et al., 2001; Langhoff et al., 2017; León et al., 2018). Current research on placental imaging is much more focused on the more advanced techniques of functional MRI (fMRI), rather than assessment of placental morphometry with conventional MRI. These fMRI techniques, and their implications for diagnosing FGR, are not in the scope of this review but are described in the reviews of Avni et al. (2015) and Siauve et al. (2015).

Although current research focuses more on the possibilities of fMRI, five studies specifically investigated the placenta morphometry measurements with MRI in relation to FGR, or markers of FGR (**Table 2**). The study of Derwig et al. was the only one that used SGA as outcome rather than FGR, and showed that small placental volume is predictive for delivering a SGA-neonates, which is in line with the findings from other (ultrasound) studies and could be a physiological phenomenon (Derwig et al., 2011). They also described that small placental volume was significantly associated with higher PI of the uterine artery, a marker of FGR. Increased uterine artery PI is thought to reflect defective trophoblast invasion, which could result in reduced placental growth (Arakaki et al., 2015).

Three studies (Damodaram et al., 2010; Ohgiya et al., 2016; Andescavage et al., 2017) investigated placental morphometry measurements in a FGR population compared to healthy controls. Although different definitions of FGR have been used (see **Table 2**), they all showed significantly reduced placental volume in the FGR population compared to the healthy pregnant population. Furthermore, Damodaram et al. showed that the placental volume remained significantly smaller throughout gestation in the FGR group, and that a lower placental volume was also associated with the severity of the FGR (detailed information on the severity subgroups can be found in **Table 2**) (Damodaram et al., 2010). Finally, Andescavage et al. described that the placental volume was significantly lower in a subgroup of the FGR-population with abnormal umbilical artery Doppler (Andescavage et al., 2017). Higher mean placental thickness, lower macroscopic placental surface area and increase in max placental thickness/placental volume (PT/PV) ratio, were placental morphometry parameters that were significantly associated with FGR (Damodaram et al., 2010; Ohgiya et al., 2016).

Further substantiation of the relevance of the max PT/PV ratio was shown by the significant correlation found between a higher max PT/PV ratio and the severity of the FGR, and the association with fetal and early neonatal morbidity in case of an increase of the max PT/PV ratio above the 95th percentile for gestation (Damodaram et al., 2010).

The last and most recent study of Dahdouh et al. had a slightly different study design. In this study placenta morphometry, although in combination with placental textural features computed on 3D MRI images, were used in two machine learning frameworks to predict FGR and BW for both healthy and FGR fetuses (Dahdouh et al., 2018). This semi-automated framework was able to detect FGR-fetuses with a diagnostic accuracy of 86%, sensitivity of 86% and a specificity of 87%. In line with the other four studies, placental volume was one of the most important features for identification of FGR. Although this study had a small sample size ($n = 80$), these results are promising, outperforming the current standard clinical tools for diagnosing FGR. Although MRI is increasingly used during pregnancy, especially at advanced gestation or in obese women (Millischer et al., 2013), availability is limited and costs are high. Therefore, current clinical use of MRI in the assessment of placental morphometry is very limited.

Postnatal Assessment of Placental Morphometry

After birth, standard placental measures are placental disk shape, diameter, surface area, disk thickness, weight, location of umbilical cord insertion site relative to the edge of the placental disk, and placental weight in relation to birth weight (Khong et al., 2016). It is advised to use placental weight trimmed of extraplacental membranes and umbilical cord (Khong et al., 2016). Inconsistencies in preparation of the placenta before weighing remains in different studies. Therefore, it is important to check whether trimmed or untrimmed placental weights are used, as for direct comparisons between absolute placental weights, values should be standardized to trimmed placental weight (Leary et al., 2003).

There is increasing evidence that features of placental gross morphology are linked biologically to the functional capacity of the placenta (Burton et al., 2016), but it has received little clinical interest. The reason for this is the timing of investigation of the placenta: pregnancy conditions have either developed or not, and intra-uterine problems already have taken place before

TABLE 2 | Literature overview of antenatal placental morphometry assessment with MRI in relation to FGR (markers).

Author, year, country	Study type and population	FGR definition	Determinant(s)	Outcome	Results
Damodaram et al., 2010, United Kingdom	Prospective 48 SP **A:** 20 FGR fetuses (1) $n = 3$, (2) $n = 3$, (3) $n = 10$, (4) $n = 1$, (5) $n = 3$ **B**: 28 controls	EFW <p5 and (1) PI UmA >p95 (2) PI UmA>p95 and PI MCA <p5 (3) AEDF in UmA (4) REDF in UmA (5) Absent or reversed "a" wave in DV and/or pulsatility in UV	PV, max PT, PT/PV ratio GA: 20–38 wks	Morphometry determinants in relation to: - Group A vs. B - Severity of FGR - Fetal and neonatal mortality	Sign. increase in max PT/PV ratio in group A Sign. correlation: max PT/PV ratio –severity FGR, PV–EFW, PV–severity of FGR Association: increase in max PT/PV ratio >p95–fetal and early neonatal mortality PV remained sign. smaller in group A
Dahdouh et al., 2018, United States of America	Prospective 49 SP **A:** 43 FGR fetuses **B:** 46 controls	EFW <p10 and - Abnormal Doppler of fetal vessels - Asymmetric growth with lagging AC	PV, PT, PL icw textural features used in machine learning frameworks GA: 18–39 wks	Identification of the FGR pregnancies	The proposed machine-learning based method using shape features identified FGR pregnancies with 86% accuracy, 77% precision and 86% recall.
Ohgiya et al., 2016, Japan	Prospective 50 SP **A:** 25 FGR fetuses **B:** 25 controls	No definition given	PT, PSA, PV GA: 19–38 wks	Morphometry determinants in group A vs. B	Sign. lower mean PSA and PV in group A compared to group B. Sign. higher mean PT in group A compared to group B.
Andescavage et al., 2017, United States of America	Prospective 114 SP **A:** 35 FGR fetuses **B:** 79 controls	EFW < p10 and - UmA PI >p95 or CPR<1 - AC lagged HC > 1 week	PV GA: 18–40 wks	Morphometry determinant in relation to: - group A vs. B - UmA Doppler, UtA Doppler, MCA Doppler, CPR	Sign. lower mean PV in group A vs. B Sign. lower mean PV in subgroup of group A with abnormal UmA Doppler. no association between: PV–UtA Doppler, PV–MCA Doppler, PV–CPR
Derwig et al., 2011, United Kingdom	Prospective 83 SP	Not applicable	PV GA: 24 - 29 wks	Morphometry determinant in relation to UtA Doppler and BW-centile	Median PV was sign decreased in pregnancies delivering a SGA-neonate (<p10) PV is sign related to the degree of uterine perfusion reflected in the PI of the UtA.

AC, abdominal circumference; CPR, cerebroplacental ratio; DV, ductus venosus; EFW, estimated fetal weight; FGR, fetal growth restriction; GA, gestational age; HC, head circumference; icw, in combination with; MCA, middle cerebral artery; PI, pulsatility index; PL, placental length; PSA, macroscopic placental surface area; PT, placental thickness; PV, placental volume; sign, significantly; SP, singleton pregnancies; UtA, uterine artery; UmA, umbilical artery; UV, umbilical vein; wks,weeks.

the possibility to investigate the placenta. However, postnatal morphology studies of the placenta give the opportunity to help in finding the neonate who suffered undetected growth restriction and should be monitored more closely during postnatal care. It is thus important to focus on the possible clinical relevance of placental morphometry in retrospectively diagnosing impaired growth.

Postnatal Placental Morphometry in Relation to Ultrasound Markers of Fetal Growth Restriction

It has been shown that utero-placental blood flow and fetal growth can be related to the gross morphometry of the placenta (Salavati et al., 2016). Small placental area and low placental weight were associated with, respectively, higher uterine and higher umbilical artery PI. Both placental area and weight were associated with a slower fetal ACGV (Salavati et al., 2016). The circularity of the placenta was associated with the uterine artery, but not the umbilical artery, flow velocity waveform. These results show that size and shape of the placenta are depending on the vascular function of the placenta from both the maternal and fetal side. Although some studies have focused on the relationship between ultrasound measurements (e.g., fetal biometry, Doppler flow velocity waveforms) and adverse pregnancy outcome (Ghosh and Gudmundsson, 2009; Sovio et al., 2015), more literature on the relationship with postnatal placental morphometry is lacking.

Birth weight to placenta weight-ratio in relation to ultrasound markers

Research showed that not only the size and weight of the placenta, but also the weight of the placenta in relation to birth weight was associated with both umbilical and uterine artery PI (Salavati et al., 2018). Specifically, high BWPW-ratio was associated with both higher umbilical artery PI (26 weeks of gestation) and higher uterine artery PI (20 weeks of gestation), two markers of decreased placental function. Low BWPW-ratio was not associated with either umbilical or uterine artery PI, however it was with maternal and neonatal morbidity (Salavati et al., 2018). Decreased placental function may sound contradictory, since placentas in the group of high BWPW-ratio can also be seen as very efficient. It might be plausible that the relatively small placentas in the group of high BWPW-ratio work at their maximum function capability for that volume, and that the birth weight actually could have been higher with higher placental volume. With this said, we would expect high BWPW-ratio to be related to adverse postnatal outcome related to starvation caused by too relatively small placentas, which was not shown by recent research (Salavati et al., 2018). This might be the result of intervention bias: those cases with high umbilical and uterine artery PI might have experienced an earlier induction of labor, resulting in lower neonatal morbidity (Gibson et al., 2014). Another explanation might be the role of placental surface area. Those placentas in the group of low BWPW-ratio might be thicker but have a small macroscopic placental surface area, resulting in a less efficiently exchange process of oxygen, nutrients, and fetal waste products. In the group of high BWPW-ratio the reverse might be true; within this group the placentas might have a large macroscopic placental surface area, but are really thin, explaining the lower placental weight.

Postnatal Placental Morphometry in Relation to Birth Weight and Fetal Growth Restriction

Various studies have looked at the relationship between postnatal placental morphometry and birth weight. These studies showed that low birth weight was associated with lower placental weight and volume, and a smaller placental area (Balihallimath et al., 2013; Kowsalya et al., 2013). Balihallimath et al. studied the relationship of placenta morphometry more specifically in different birth weight groups, classified by gender (Balihallimath et al., 2013). In the groups with birth weight less than 3,000 g, the surface area of the placenta was smaller in male babies compared to female babies. When birth weight exceeded 3,000 g, the surface area was larger in male babies (Balihallimath et al., 2013). In addition, it has been described that male babies have a higher birth weight compared to female babies, but have the same placental weight (increased BWPW-ratio) (Eriksson et al., 2010; Macdonald et al., 2014). Male babies also have a higher perinatal mortality (Drevenstedt et al., 2008), rather than more efficient they may also be just the fetus that runs the risk of increased mortality to maximize growth. We think the placenta has a functional reserve to cope with extra demands, which explains why many stillbirths have pre-existing injury (Gardosi et al., 2013).

Although a small placenta suggests reduced reserve, the association between low birth weight and low placental weight and size, could potentially be physiological (small baby, small placenta) therefore statements regarding the association between placental morphometry and pathological, low or high, birth weight, require an investigation of proportionality, of the relationship of placental morphometry with BWPW-ratio. Also the site of the umbilical cord insertion has been linked to birth weight (Yampolsky et al., 2009; Haeussner et al., 2013; Kowsalya et al., 2013). Kowsalya et al. indicated that the cord insertion was more often eccentric or marginal in the group of infants with low birth weight (Kowsalya et al., 2013). Yampolsky et al. pointed out that this central insertion influences placental efficiency positively (Yampolsky et al., 2009). Conflicting results were published by Haeussner et al. who reported that the location of the cord insertion in relation to the edge of the placental disk did not correlate with birth weight, and eccentric cord insertion did not necessarily compromise efficiency of the normal human placenta (Haeussner et al., 2013). Furthermore, they reported that parameters regarding the form of the placenta (e.g., diameter, thickness, roundness, eccentricity of the cord insertion) correlate with both birth weight and placental weight (Haeussner et al., 2013).

It has been proposed that FGR and morphologic changes of the placenta, are caused by impaired placental perfusion, due to reduced placental vascular bed in chronic fetal hypoxia, which causes oxidative stress of the fetal vasculature (Kingdom and Kaufmann, 1997; Kuzmina et al., 2005). Junaid et al. investigated micro and microvasculature of placentas from normal and FGR pregnancies, and observed hyposvascularity in the peripheral lobules of placentas from FGR pregnancies (Junaid et al., 2014).

TABLE 3 | Literature overview of postnatal placental morphometry assessment in relation to FGR (markers).

Author, year, country	Study type and population	FGR definition	Determinant(s)	Outcome	Results
Egbor et al., 2006 United Kingdom	Prospective **A:** 17 FGR **B:** 16 PE-FGR **C:** 16 controls	- Clinical evidence of suboptimal growth - Ultra sonographic evidence of deviation from appropriate growth percentile - BW <p10	PV, PW	FGR	FGR was associated with a significant reduction in PV and PW
Mayhew et al., 2007, United Kingdom	Prospective **A:** 5 FGR **B:** 5 PE-FGR **C:** 9 controls	- Deficient fetal growth on ultrasound scans - IBR <p10	PSA	Morphometry determinant in the different groups	FGR (with or without PE) was associated with a reduced PSA
Almasry and Elfayomy, 2012, Saudi Arabia	Case-control **A:** 50 FGR **B:** 25 controls	EFW<p10 icw two criteria: - Abnormal UmA Doppler - Oligohydramnios; AFI<5 - Asymmetric growth; HC/AC ratio	PD, PW, "placenta co-efficient" (PW/BW).	FGR	Significant reduction in PD and PW in FGR group as compared with controls. Placental coefficient greater in FGR group.

AC, abdominal circumference; BW, birth weight; EFW, estimated fetal weight; FGR, fetal growth restriction; HC, head circumference; IBR, individualized birth weight ratio (is calculated using factors including fetal gender, GA, parity, ethnic origin and maternal age, height and booking weight); icw, in combination with; PE, preeclampsia; PD, placental diameter; PSA, macroscopic placental surface area; PV, placental volume; PW, placental weight; UmA, umbilical artery.

Another aspect that may result in altered morphometry, is the fact that FGR related hypoxia influences angiogenesis via various growth factor receptors (Mayhew et al., 2004). Three studies were found that investigated the relationship between postnatal placental morphometry and FGR (Egbor et al., 2006; Mayhew et al., 2007; Almasry and Elfayomy, 2012) (**Table 3**).

These three studies classified FGR based on deficient fetal growth on ultrasound scans and EFW less than 10th centile. They found that FGR was associated with changes in placental morphometry such as decreased surface areas, decreased placental diameter, and decreased placental volume and placental weight (Egbor et al., 2006; Mayhew et al., 2007; Almasry and Elfayomy, 2012).

FUTURE PERSPECTIVES

As described, various studies have focused on the relationship between placental morphometry and fetal growth and birth weight. Unfortunately, only limited research has been performed focusing on associations between placental morphometry and FGR. The often seen "SGA-FGR confusion" in FGR studies will lead to weaker associations with any effective screening test, including placental morphometry imaging. Although cheap and easy to obtain, the postnatal placenta will only aid the diagnosis of FGR in retrospect. This could result in altered management by less monitoring in healthy SGA and more monitoring in FGR, regardless of birth weight. Although we expect that aspects of placental morphometry can play a role in the diagnostic process of FGR, mainly postnatally, additional components, as part of a multiparameter model, might need to be taken into account with the aspects of placental morphometry. A small placenta, or abnormally flat or thick placenta may prompt review for assessment of the baby and the histology of the placenta to see if other findings to suggest maternal malperfusion or other pathology is present.

Further use of placenta morphometry in the diagnostic process of FGR can be explored by for example BWPW-ratio in relation to abdominal circumference growth velocity (ACGV), macroscopic placental surface area (and placental volume) and placenta serum biomarkers. As suggested in a recent paper about screening for fetal growth restriction (Gaccioli et al., 2018a), it is expected that future screening tests for FGR will include several measurements, which are obtained from both imaging procedures and measurements of biomarkers. For the development of such a model it is essential that every single parameter is measured and scaled in the association with FGR, in order to generate consistent associations. Regarding imaging procedures, research has shown that placental imaging through MRI might be of clinical use in predicting FGR. Nowadays, MRI is already in use for fetal imaging, so possibly placental imaging can be used in the near future.

CONCLUSIONS

It is of great importance that clinically useful, and easy to perform, methods will be generated in order to improve the

antenatal and postnatal screening for, and diagnosis of, fetuses with FGR who have increased risk on adverse pregnancy outcomes. In this literature review we intended to give an overview on the clinical relevance of placenta morphometry in the detection of FGR. In current clinical practice, antenatal placental imaging is difficult, and the placenta is not routinely examined after birth, nor in a standardized way, despite the possible value in several parameters of impaired growth of the fetus. Future research can focus on the relationship between placental morphometry, FGR and its complications, to improve screening for FGR, and to determine the biological pathways that can be linked to placental dysfunction, in a group of optimally phenotyped cases of FGR. With this, placental morphometry might be implemented in clinical practice, possibly as part of a multiparameter model.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception of this review and the critical appraisal of the literature summarized herein. NS and MS generated the initial draft of the manuscript, and this was critically revised by SG, WG, AC, TP, and JE. All authors approved the final version and submission of the article.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 07 February 2019
doi: 10.3389/fendo.2019.00055

Neonatal Morbidities of Fetal Growth Restriction: Pathophysiology and Impact

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Being born small lays the foundation for short-term and long-term implications for life. Intrauterine or fetal growth restriction describes the pregnancy complication of pathological reduced fetal growth, leading to significant perinatal mortality and morbidity, and subsequent long-term deficits. Placental insufficiency is the principal cause of FGR, which in turn underlies a chronic undersupply of oxygen and nutrients to the fetus. The neonatal morbidities associated with FGR depend on the timing of onset of placental dysfunction and growth restriction, its severity, and the gestation at birth of the infant. In this review, we explore the pathophysiological mechanisms involved in the development of major neonatal morbidities in FGR, and their impact on the health of the infant. Fetal cardiovascular adaptation and altered organ development during gestation are principal contributors to postnatal consequences of FGR. Clinical presentation, diagnostic tools and management strategies of neonatal morbidities are presented. We also present information on the current status of targeted therapies. A better understanding of neonatal morbidities associated with FGR will enable early neonatal detection, monitoring and management of potential adverse outcomes in the newborn period and beyond.

Keywords: IUGR, FGR, bronchopulmonary dysplasia, cardiac, brain injury, necrotizing enterocolitis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *30 October 2018*
Accepted: *22 January 2019*
Published: *07 February 2019*

Citation:
Malhotra A, Allison BJ, Castillo-Melendez M, Jenkin G, Polglase GR and Miller SL (2019) Neonatal Morbidities of Fetal Growth Restriction: Pathophysiology and Impact. Front. Endocrinol. 10:55. doi: 10.3389/fendo.2019.00055

OVERVIEW AND DESCRIPTION

Fetal growth restriction (FGR) describes the fetus that does not grow to its expected biological potential *in utero*, and is a relatively common complication of pregnancy. True FGR, as compared to constitutional smallness, is a pathological condition wherein the placental fails to deliver an adequate supply of oxygen and nutrients to the developing fetus, termed placental insufficiency. As a consequence, fetal growth becomes stunted. It is only in the last several years that consensus definitions for pathological FGR have been developed (1), but it remains that many cases of FGR *in utero* remain undetected, and therefore the neonatal description of small for gestational age (SGA) continues to be a useful and necessary proxy for FGR (2). Traditionally, an estimated fetal weight or abdominal circumference of less than the 10th centile for the population at a given gestational age was considered highly suggestive of FGR. However this broad description of SGA includes the many infants (∼20%) that are born small, but are otherwise healthy (2). Accordingly, consensus definitions for FGR now incorporate Doppler indices of placental function/ dysfunction during pregnancy (1), to provide a more robust assessment of pathological fetal growth restriction.

Clear and well-defined guidelines for description of FGR subsequent to placental insufficiency are important for two broad reasons, (i) early identification of FGR flags infants who are at significantly elevated risk for neonatal complications, and (ii) early identification of infants with FGR who would benefit from intervention(s) to improve outcomes. The etiology of many adverse consequences of FGR arise *in utero* from fetal hypoxia and nutrient deprivation secondary to placental dysfunction, with fetal hemodynamic adaptations *in utero* laying the foundation for altered organ structure and function in the neonatal period and beyond.

ETIOLOGY AND UTEROPLACENTAL FACTORS

The basic determinants of fetal growth are the individual's genetic makeup, nutrient availability from the mother, and environmental factors, coupled with the capacity of the placenta to adequately transfer nutrients and oxygen to the fetus, and endocrine modulation of these interactions (3, 4). Reduced fetal growth, and subsequent pathological FGR, can be caused by maternal factors (e.g., under nutrition, hypertension, preeclampsia), fetal (chromosomal abnormalities, multiple fetuses) or placental factors (5), however in the majority of cases, FGR results from placental dysfunction (6). Here, the term *placental insufficiency* is broadly used to describe reduced transfer of oxygen and nutrients to the fetus, with adverse effects on fetal development. Antecedents of placental insufficiency can include maternal malnutrition and hypertension, but in up to 60% of cases the placental insufficiency is idiopathic, wherein there is a physiological deficiency in the remodeling of uterine and placental spiral arteries resulting in restricted uteroplacental perfusion (7).

Abnormalities in placental function provide a primary clinical indicator that transfer of oxygen and nutrients is suboptimal, and fetal growth may be adversely affected. In the fetus, placental insufficiency is characterized by preferential blood flow redistribution to the vital organs (brain, myocardium, and adrenal glands), while other organs, including the gastrointestinal tract, skin, and others may be deprived of sufficient blood flow. This fetal redistribution of blood flow occurs as a direct result of hypoxia, and can be detected as altered umbilical, uterine and/or middle cerebral artery Doppler flows (8). Large population studies of small but otherwise healthy infants at birth (Apgar $\geq$ 7 at 5 min of life) demonstrates that severely growth restricted infants at the third birth weight centile are indeed chronically hypoxic; umbilical vein median pO_2 13 mmHg (FGR) versus 26 mmHg (normally grown infants), and median SaO_2 16 vs. 55% respectively (9, 10).

In addition to the fundamental roles of oxygen and glucose for development, fetal growth is dependent on a number of key anabolic hormones—placental, pancreatic, thyroid, adrenal and pituitary hormones—any disruption in these can also lead to FGR (11, 12). The insulin-like growth factors -I and -II (IGF-I and IGF-II) are both proposed to play central roles in normal fetal growth, stimulating fetal cell proliferation, differentiation, protein and glycogen synthesis, where these actions are mediated via their receptors and the IGF-binding proteins (IGFBPs). The two IGFs are detected in the fetal circulation in early gestation, and in particular it is noted that decreased serum IGF-1 is correlated with reduced fetal growth (3, 13). IGF-1 also has a central role in brain growth, white matter development and brain connectivity (14). Pregnancy-associated plasma protein-A (PAPP-A), secreted by the placental decidua, cleaves IGFBP-4, which in turn is a potent inhibitor of IGF bioactivity. Accordingly, low levels of PAPP-A in early pregnancy are linked with an increased risk for FGR, although the predictive value of this biomarker still remains poor (15). A recent study has investigated whether administration of IGF-1 into the amniotic fluid can improve postnatal growth and metabolism in a sheep model of FGR, and results from this study look promising (16) (see Interventions for Improved Outcomes section). Glucocorticoid hormones play a central role in the development and maturation of fetal organs, while growth hormone, which is the major hormonal regulator of postnatal growth, has no demonstrable effect on fetal growth *per se* (17). Exogenous glucocorticoids are administered to pregnant women at imminent risk of preterm birth to mature the fetal lungs, and preterm birth is a common complication of FGR. Preclinical and clinical evidence demonstrates that antenatal steroids may exacerbate growth restriction (particularly repeat doses) (18) and that the FGR fetus differentially responds to antenatal steroids compared to appropriately-grown fetuses, likely mediated via altered placental response to steroids (19). Antenatal glucocorticoids may not significantly improve neonatal outcomes in FGR preterm infants (20), and indeed, may have adverse effects on brain development (21, 22). Further research is clearly needed in this area.

The fetus mounts a critical hemodynamic response to hypoxia, aimed at ensuring the most important fetal organs maximize their oxygen supply. This adaptive response redistributes blood flow away from peripheral vascular beds which is preferentially shunted toward essential organs, termed *brain sparing* (23). This results in preferential supply of blood flow to favor the brain, heart, and adrenals, at the expense of the gut, kidney, hematologic organs, and peripheral vascular beds. When fetal hypoxia is chronic in nature, as occurs with placental insufficiency, the persistent fetal hemodynamic shift has significant consequences for the fetus and neonate. Characteristically, prolonged fetal hypoxia reduces fetal weight overall, but also does so in an asymmetric manner, with relatively spared head size and a thin and/or shorter body length. While hemodynamic redistribution may be an attempt to protect vital organs from hypoxic injury, an adverse impact on fetal organ development and vascular remodeling is increasingly being recognized (23, 24). For example, the shunting of blood flow away from the kidneys is now recognized as contributing to suboptimal renal development with reduced nephron endowment (25). Further, sustained vasoconstriction of peripheral vascular beds alters local arterial wall properties including endothelial vasodilator dysfunction and sympathetic hyperinnervation, and consequently contributes to cardiac remodeling (26). The short and long-term consequences of sustained redistribution of cardiac output are profound, for

both spared and non-spared organs, and these will be discussed in more detail below.

The overall incidence of FGR depends on the diagnostic criteria used, and the population being examined. It is estimated that between 3 and 9% of pregnancies in the developed world, and up to 25% of pregnancies in low-middle income countries are affected by FGR (27, 28). Factors that influence FGR rates in communities include maternal nutrition, maternal and paternal smoking rates, alcohol and drug addiction, socio-economic status, maternal activity, stress during pregnancy and genetic make-up (29). The incidence of FGR is significantly higher in low- and middle- income countries, compared to high-income countries, and this is notably contributed by a large number of FGR infants born in the Asian continent, which accounts for approximately 75% of all affected infants in the world, followed by Africa and South America (30).

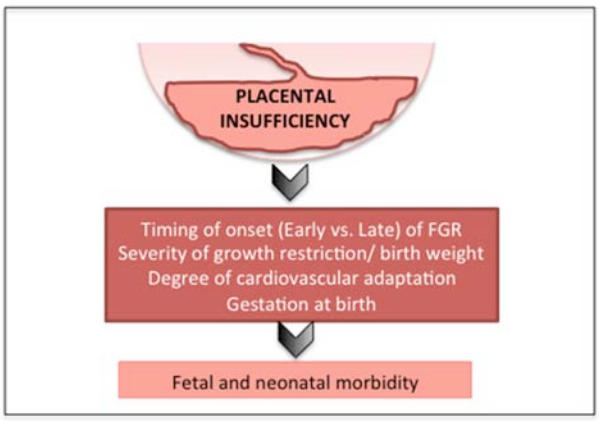

FIGURE 1 | Factors that affect outcomes in FGR.

CLASSIFICATION TYPES OF FGR

FGR can be classified as early- or late-onset, reflecting the gestational age when growth restriction is diagnosed. Early onset FGR (<32 weeks gestation) is the more severe phenotype, associated with significant disruption to placental perfusion leading to chronic fetal hypoxia, and with subsequent fetal cardiovascular adaptation *in utero* (31). Fetuses with early-onset placental insufficiency are more likely to be born preterm, to deteriorate over weeks, and have a high risk of morbidity or mortality. Late onset FGR (≥32 weeks gestation) is the more common presentation of growth restriction (up to 80% of FGR cases), and is generally linked with a milder placental deficit, together with a lesser degree of fetal hemodynamic adaptation. Although placental dysfunction is mild, this group has a high risk of deteriorating rapidly, such that they have an elevated risk of stillbirth (31). This broad distinction between early- and late-onset FGR demonstrates that the timing when placental function becomes rate limiting for the fetus is a principal factor affecting outcome.

Advances in obstetric monitoring mean that it is increasingly likely that placental insufficiency and fetal growth restriction are detected during pregnancy. However, a significant proportion (up to 50%) of FGR fetuses remain undiagnosed, and are first recognized only very late in pregnancy or at birth (32–34). Furthermore, debate continues around the utility of third trimester ultrasound for the detection of late-onset FGR (35), with a recent study reporting that undiagnosed FGR does not lead to increased incidence of morbidity in neonates (36). These data likely reflect that it is predominantly the early-onset FGR infants with severe placental insufficiency, and worse neonatal outcomes, who are more straightforward to detect during pregnancy. Currently, no effective antenatal therapy exists for FGR, hence, delivery of the fetus remains the only viable option for a severely affected pregnancy; this often occurs preterm, introducing further risk of morbidity and mortality (37, 38). Together these data are indicative that the timing of the onset of placental insufficiency (early vs. late), gestation at birth, and severity of compromise/birth weight are the most predictive factors for neonatal outcomes (39) (**Figure 1**).

PERINATAL MORBIDITIES

A typical FGR infant at term age and an appropriately grown infant at term are shown in **Figure 2**. Key pathophysiological mechanisms driving fetal growth restriction and the resulting *in-utero* and postnatal consequences are highlighted in **Figure 3**. Placental pathology and FGR are strongly associated with fetal demise *in utero*, and stillbirth (40–42). FGR is the greatest risk factor for stillbirth; overall it is shown that up to 50% of infants who are stillborn were small for gestational age or growth restricted (43). The detection, early diagnosis, surveillance and delivery of the severely growth restricted fetus are paramount to decrease stillbirth, but it remains that 40% of severe FGR infants (<3rd centile for birth weight) remain undetected *in utero* (44).

After birth, FGR infants are more likely to spend a significantly longer time in NICU compared to gestation age-matched infants (45). Accordingly, financial costs associated with the care of FGR infants are high, given that many of them will remain in NICU for prolonged periods (46, 47). FGR infants demonstrate elevated rates of intolerance to feeds/ milk, feeding difficulties and necrotizing enterocolitis (NEC). NEC is predominantly seen in infants who are born preterm, but late preterm infants are more likely to develop NEC if they were growth restricted (48). It is likely that i*n utero* chronic fetal hypoxia and subsequent cardiovascular redistribution of blood flow away from the gastrointestinal tract contribute to immature gut development (49). FGR newborns, especially with abnormal flows in the umbilical artery prior to birth, are shown to have more feed intolerance when compared to their well-grown preterm counterparts (50). Superior mesenteric artery blood flows have been used as a marker for splanchnic perfusion in neonates and decreased flows correlate with feed intolerance (51). Application of near infra-red spectroscopy in the neonatal period as an assessment tool for monitoring gut perfusion can detect changes in splanchnic oxygen delivery, which may be reduced in FGR

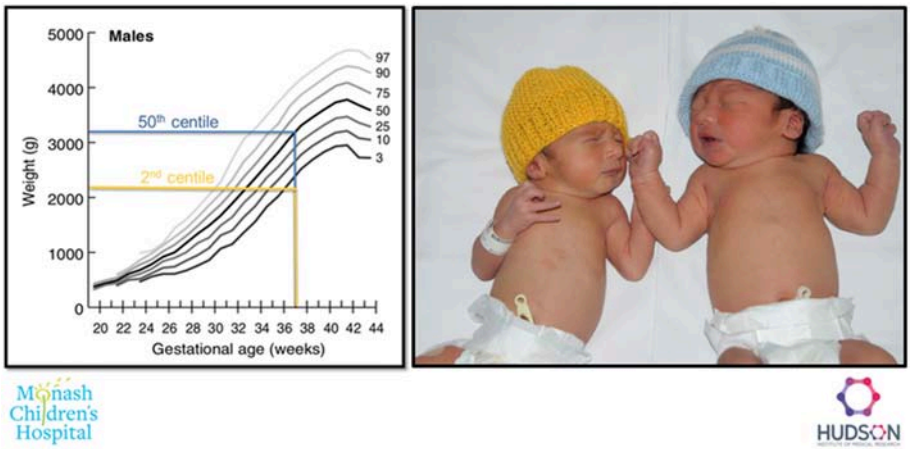

FIGURE 2 | Example of a FGR (2nd centile weight for age, yellow), and an appropriately grown (50th centile weight for age, blue) infant born at 37 weeks gestation.

infants and may predict feeding intolerance and development of NEC (52). Studies have shown that preterm FGR infants do not tolerate enteral feeds in the first few days of life (53) but conversely there is evidence that delaying enteral feeds in preterm FGR infants does not confer any protection against feed intolerance or NEC (54). In fact, it may delay establishment of feeds and increase length of stay in the neonatal unit (55).

Malnutrition and low birth weight puts FGR infants at an increased risk of a number of transient neonatal morbidities including hypothermia, altered glucose metabolism (hypoglycemia, hyperglycemia), hypocalcemia, polycythemia, jaundice and sepsis (5). Increased risk of infection is also common, potentially related to depressed immunological state and competence (56). FGR infants born preterm also have an increased risk of retinopathy of prematurity (57). FGR is linked to altered nephrogenesis, due to suboptimal tubular development caused by intrauterine hypoxia (58), and in turn, urinary Cystatin-C excretion is increased in FGR infants compared to appropriately-grown infants which is seen to reflect reduced renal volume (59). It is therefore suggested that increased secretion of Cystatin-C signifies nephron loss as a result of the negative impact of FGR on kidney development. Factors involved in nephron loss may include intrauterine hypoxia, decreased antioxidant capacity, and altered levels of growth factors.

SPECIFIC NEONATAL MORBIDITIES (TABLE 1)

Cardiovascular Morbidity

Clinical Features

In addition to chronic hypoxia, placental insufficiency imposes other important stressors for the developing fetus, such as oxidative stress, inflammation and increased hemodynamic stress. This leads to elevated cardiac afterload due to high placental vascular resistance, which in turn directly and indirectly impacts on the developing cardiovascular system. It is now accepted that the fetal adaptations to these combined stressors sets the fetus, and future offspring, on a path of predetermined increased risk of cardiovascular disease (60, 61). It is also now apparent that subclinical or subtle evidence of cardiovascular dysfunction is present in fetal and/or early neonatal life, well before the onset of significant cardiovascular or metabolic disease in adulthood, supporting the notion of perinatal programming (60).

Advances in Doppler ultrasonography of the placental and fetal circulations provide a window of opportunity to observe and quantify fetal cardiovascular function, and early dysfunction. In early-onset FGR, severe placental insufficiency is characterized by high vascular resistance within placental vascular beds, resulting in absent or reversed diastolic umbilical artery flow, as well as high pulsatility index in the ductus venosus and increased dilation of cerebral vessels evident of fetal brain sparing (31). In late-onset FGR, umbilical artery flow may be normal, representing a milder placental insufficiency. Despite this, brain sparing is still evident, with increased cerebral to placental blood flow driven primarily by vasodilation within the middle cerebral artery in response to hypoxia (62). In both early- and late-onset FGR, increasing vasodilation within cerebral vascular beds is indicative of a worsening fetal state (63). Increased myocardial performance index, an index incorporating both diastolic and systolic function to assess global cardiac function/dysfunction, is evident from 24 weeks gestation in early onset-FGR fetuses (64). An increase in myocardial performance index is not indicative of improved performance, but rather demonstrates an increased time of systolic relaxation evident in early-onset FGR. Increased myocardial dysfunction is also present in late-onset FGR from >35 weeks gestation. In this population, late-onset placental insufficiency and FGR results in fetuses with larger, more globular hearts and early indices of functional deficits with impaired relaxation (65). This study is the first to show that late-onset placental insufficiency and FGR induces cardiac dysfunction that is detectable in the third trimester of pregnancy (∼35 weeks gestation), indicating the presence of cardiac programming prior to birth. It has also been shown that

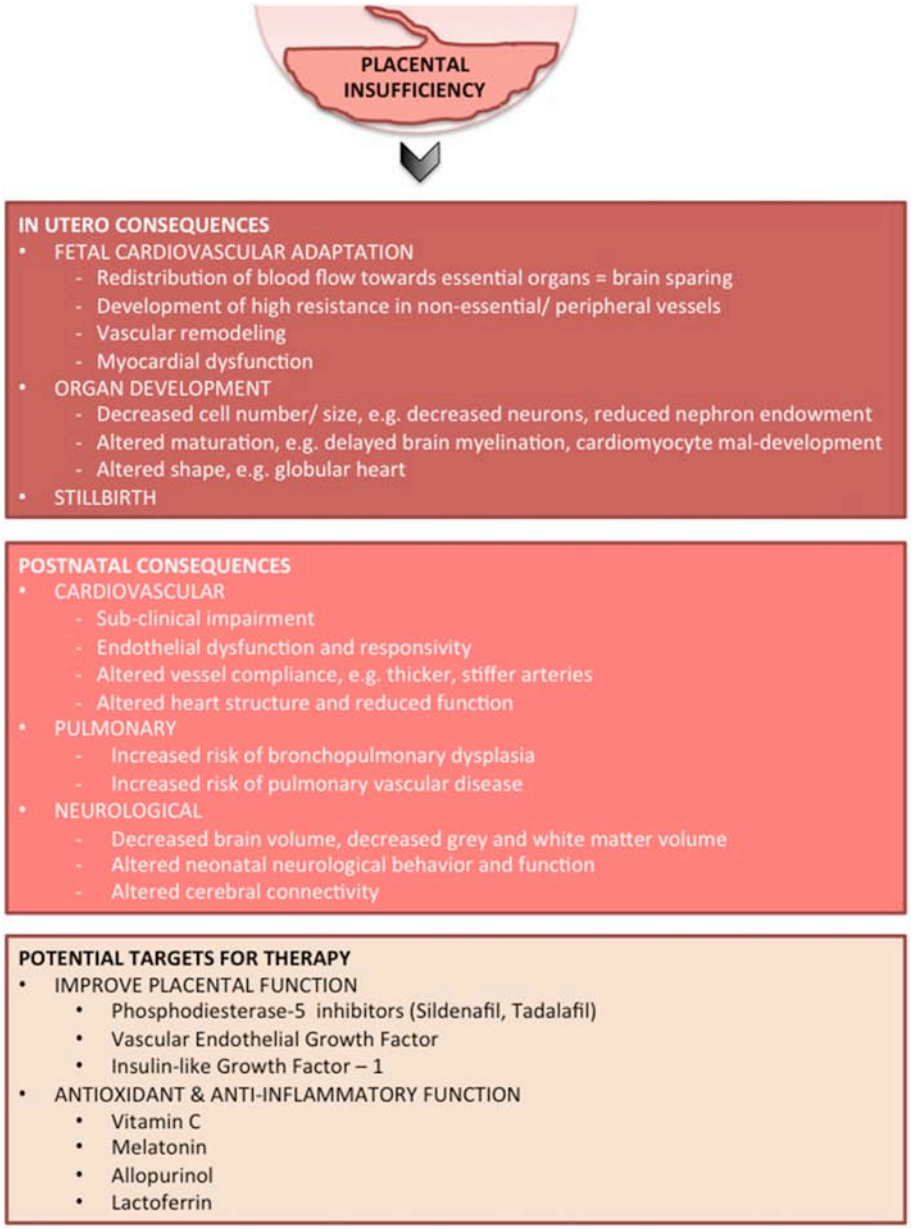

FIGURE 3 | *In-utero* and postnatal consequences of FGR and potential targeted therapies.

cardiac dysfunction and markers of cardiac injury such as BNP and H-FABP become increasingly worse as the severity of fetal compromise progresses (66).

Pathophysiology

In the presence of very high placental resistance associated with a sub-optimal pregnancy, the fetal heart contracts against an increased afterload, thereby increasing the work required to contract with each beat, resulting in increased heart wall stress and hypertrophy (65). Over a sustained period, hypertrophy increases wall thickness altering ventricular compliance. Increased afterload is evidenced by the presence of increased serum B-natriuretic peptide in infants born growth restricted (67).

Where placental insufficiency is present, the fetal heart must also adapt to a reduced supply of glucose, with the fetal heart producing ATP from glycolysis and oxidation of lactate. Despite this, cardiac glucose consumption is not altered in growth restriction due to increase in insulin receptor GLUT4 in the heart, which increases insulin transport to maintain glucose consumption (68). Thus, glucose availability is not considered a primary limiting factor for fetal cardiac function. More in-depth analysis of the effects of suboptimal oxygen and glucose supply to the developing heart can be examined

TABLE 1 | Neonatal morbidities in fetal growth restriction.

	Cardiovascular morbidity	Respiratory morbidity	Neurological morbidity	Others
Neonatal period	Early hypotension Persistent fetal circulation/PPHN Structural heart changes Vessel wall rigidity Cardiac function issues Late systemic hypertension Secondary pulmonary hypertension	Increased need for respiratory/ventilator support Meconium aspiration syndrome Pulmonary hemorrhage Bronchopulmonary dysplasia	Perinatal asphyxia Microcephaly Cranial ultrasound abnormalities (IVH, PVL) White matter and gray matter changes on MRI Functional and DTI MRI changes General movement assessment abnormalities EEG abnormalities	Poor transition Hypoglycemia Hypocalcemia Hypothermia Sepsis Jaundice Polycythemia Prolonged NICU stay Feed intolerance Delay in establishment of feeds Necrotizing enterocolitis Renal tubular injury Retinopathy of prematurity
Long term impact	Hypertension Ischemic heart disease Stroke Atherosclerosis	Chronic respiratory insufficiency Reactive airway disease	Neurodevelopmental issues Behavioral problems Learning difficulties Cerebral palsy Dementia Mental health issues	Failure to thrive Obesity Immune dysfunction Osteoporosis Metabolic syndrome Renal issues Hormonal issues Cancer Shortened life span

PPHN, persistent pulmonary hypertension; IVH, intraventricular hemorrhage; PVL, periventricular leukomalacia; MRI, magnetic resonance imaging; DTI, diffusion tensor imaging; EEG, electroencephalography; NICU, neonatal intensive care unit.

in animal studies of FGR (69). These studies show that the fetal heart is remodeled in a manner similar to that seen in dilated cardiomyopathy. Cardiomyocyte development is adversely affected and programmed cell death is increased in growth restricted fetal guinea pigs and sheep, with a persistence of the mononucleated, primitive cell type (70, 71). Permanent alterations in heart morphology are detected into adulthood, as evidenced by persistence in the deficits in cardiomyocyte number and cardiac hypertrophy (70).

The transition to ex utero represents a particularly critical period where the heart must rapidly adapt to new pressure and flow demands. After birth, the external pressures around the heart are reduced, due to alleviation of the liquid-filled lungs and amniotic fluid. Concurrently, the low resistance placental circulation is removed, temporarily decreasing cardiac output and increasing afterload, and thus heart rate, end-diastolic pressure and stroke volume must all be increased to maintain adequate cardiac output. In response to these altered pressure demands throughout the transition to ex-utero life, the myocardium undergoes rapid changes in cardiac muscle protein expression (72). One critical change precipitated by such pressure changes at birth is a shift in the fibrous component of sarcomeres toward smaller isoforms, which increase the passive tension within with postnatal heart (72). It is postulated that the growth-restricted fetus undergoes these changes *in utero*, due to the presence of increased afterload secondary to high placental resistance, and resulting in altered cardiac compliance (73). In human infants and in experimental animal models of FGR, the heart is shown to have shorter sarcomeres, which likely contributes to decreased contractile strength (73, 74). Further, changes in the large sarcomere protein titin are described in the FGR heart, reflecting a shift from a large compliant isoform toward a small and stiff isoform (73). As titin is a major determinant of sarcomere length, this change in isoform is consistent with overall reduction in sarcomere length in the hearts of growth-restricted fetuses, and has consequences for cardiac development and function.

Changes in the hearts of growth-restricted fetuses are directly coupled with changes in the wider cardiovascular system, notably the vasculature. It is now well described that vascular responses to placental insufficiency and chronic hypoxia are vascular bed-dependent. In peripheral vascular beds, human and animal data show that sustained vasoconstriction and peripheral vascular resistance in response to chronic hypoxia induces arterial stiffness and elevated central pulse pressure (75–77). Growth restriction induced via chronic hypoxia increases peripheral vascular tone via numerous methods, including endothelial dysfunction (76), increased sympathetic nervous system activation (69) and oxidative stress (78). Oxidative stress, induced via increased reactive oxygen species generation, quenches nitric oxide (NO), thereby reducing its bioavailability and increasing peripheral vascular tone. We have previously described an increase in plasma urate levels arising from chronic fetal hypoxia (78) suggesting activation of a potent oxidative enzyme, xanthine oxidase. Importantly, it is this altered vascular tone in fetal life that sets up developmental programming for future hypertension, as evidenced in both FGR animals (79) and human cohorts (61). Central vessels, such as the aorta and carotid arteries, have increased wall thickness (80) and increased stiffness (81) in FGR humans and animals. The vascular changes described above persist into adulthood, however, they are more

pronounced in peripheral vascular beds compared to central vascular bed (82).

Vascular compensation is observed in FGR offspring, wherein remodeling of the arterial wall, collagen and elastin content contribute to altered vascular mechanics (83). Rodent and guinea pig studies show that interruption of fetal growth in mid gestation coincides with a crucial period of elastin production within vasculature, attenuating elastin deposition and subsequently content, such that elastin is reduced and collagen increased (84, 85). This remodeling greatly impacts on vascular mechanics, as collagen is 100-times stiffer than elastin and, as a consequence, vascular stiffness is significantly increased (85). These changes in vessel biomechanics are most notably in the lower body arteries of growth-restricted offspring (83). Following low protein diet restriction, the aorta from adolescent rodents are not only stiffer, they also have increased fibrotic tendency, despite being normotensive (67). However, a more profound effect on vascular extracellular matrix remodeling is seen with placental dysfunction-induced growth restriction, compared to other factors such as diet (high fat) or fetal sex. These data are suggestive that vascular remodeling occurs primarily in response to changes in pressure and flow caused by chronic hypoxia and adaptive hemodynamic redistribution, rather than metabolic or hormone alterations.

Impact

The evidence presented above all indicates that exposure to placental insufficiency and chronic hypoxia significantly alters *fetal* development of the cardiovascular system. Unsurprisingly, the fetal cardiovascular alterations subsequent to placental insufficiency persist into clearly detectable structural and functional changes in the early postnatal period. After birth, tissue Doppler imaging (TDI) has allowed detection of persistent sub-clinical changes in movement and timing of the myocardium throughout the cardiac cycle, in particular during myocardial relaxation (86). In the first days of life, infants who were growth restricted show altered cardiac structure detectable on ultrasound with decreased sphericity index (a more globular shape), together with increased interventricular septum and left ventricle wall thickness (77). Further, load-dependent diastolic function is impaired (77, 87) this often represents impaired cardiac relaxation resulting in the transition from contraction to relaxation occurring prior to aortic valve opening, a situation which is common in hearts exposed to chronically high afterload. Frequently, FGR does not alter overall cardiac output, however components of cardiac output are altered with decreased stroke volume and increased heart rate often presenting in the FGR newborn (86). With increasing severity of FGR there is increased biomarkers of myocardiac cell damage, such as heart fatty acid binding protein (H-FABP), and incremental worsening of both systolic and diastolic dysfunction and in particular heart relaxation is altered (66). These alterations are indicative of hemodynamic compromise and are linked to worsening outcomes including fetal demise (88). Early signs of alteration in blood pressure in association with FGR remains contentious—we have documented increased blood pressure in the early postnatal period (80), whilst others show no change in blood pressure (82); these differences may reflect the difference between clinical and pre-clinical studies or the severity of the growth restriction induced.

In turn, *in utero* cardiac and vascular remodeling in FGR neonates programs for cardiovascular disease into adulthood. Indeed, the consequences of growth restriction on adult cardiovascular function are now well studied, and are central to the Developmental Origins of Health and Disease (DOHAD) hypothesis. These findings are apparent from both human epidemiological and experimental paradigms in growth-restricted offspring (76, 85) and adults (60). Long-term evidence of the link between low birth weight and developmental programming is available in the infants born in famine conditions in Europe in the 1900s wherein SGA is linked with significantly higher blood pressure in later life (89), and with increased risk of ischemic heart disease and cerebrovascular disease (90). Precursors of long-term suboptimal outcomes such as stroke and hypertension (91) have been proposed to be evident in growth restriction offspring as pre-atherosclerotic vascular damage in both newborns (92) and 18 month old FGR offspring (93).

Despite excellent evidence of the link between FGR and adult cardiovascular disease, there is some difficulty in dissociating the potentially separate effects of placental insufficiency/FGR and preterm birth. Growth restricted fetuses are often born preterm, particularly early-onset severe FGR infants, and preterm birth is also associated with adverse effects on the developing cardiovascular system (94). A recent study by Cohen et al. (87) followed both preterm and preterm FGR infants to 6 months of age to determine cardiac morphology. They found that changes in cardiac structure and function associated with preterm birth alone were sub-clinical, and normalized in childhood, while only thickened ventricular walls persisted into 6 months of age in FGR infants (87). This study goes some way to delineate the separate effects of prematurity and growth restriction and suggests a possible persistence of structural changes in FGR over and above the effects of prematurity.

Respiratory Morbidity

Clinical Features

There is heterogeneity in descriptions of pulmonary complications associated with FGR, which probably reflect the heterogeneity in growth restriction itself. There is however good evidence that chronic hypoxia associated with FGR interrupts normal pulmonary development, and increases susceptibility to both short- and long-term respiratory compromise. Preterm FGR newborns are 45% more likely to have bronchopulmonary dysplasia (BPD) or die from respiratory complications after birth as compared to well-grown infants (45). Further, even FGR infants born at term have worse respiratory outcomes than appropriately grown infants (95). FGR infants spend a significantly increased time in NICU and on mechanical ventilation compared to age-matched control infants, and rates of respiratory distress syndrome (70) and BPD are increased with FGR (45, 96, 97). Indeed, large multicenter trials for early-onset FGR describe that BPD is the most common morbidity for this population. The risk of BPD is greater when FGR and preterm

birth are co-morbidities. Growth restriction is also associated with pulmonary hypertension of the newborn (96). FGR is associated with impaired lung function in children (98) that can persist to adulthood (99).

Pathophysiology

Human FGR cohort studies and preclinical animal studies describe that FGR can result in altered lung development; in some cases these are subtle structural and/or biochemical changes, wherein the timing and severity of compromise modulates effect. In animal studies, an early onset placental or hypoxic compromise mediates a more pronounced adverse outcome. Chronic hypoxia in fetal sheep resulting in FGR induces an adaptive response within the developing lung, where genes regulating hypoxic signaling, lung liquid reabsorption and surfactant maturation are increased (100). A 2-week exposure to hypoxia alone in rats disrupts alveolarization, reducing alveolar number via reduced septation (101). In fetal sheep we have induced late-onset placental insufficiency and FGR to examine lung morphology in preterm and term-born lambs. Lambs born naturally at term have simplified lung architecture with decreased secondary crest abundance and increased elastin deposition (102). Lambs that are delivered preterm and exposed to 2 h of mechanical ventilation do not demonstrate a difference in lung structure between FGR and appropriately-grown lambs, with no difference in the ratio of lung tissue to airspace or septal crest density, however the early tissue injury marker cyr61 is significantly increased in FGR lambs (103). Further, we observed that both FGR and appropriately grown lambs had similar ventilation requirements in the first hours of life. These findings extend previous results in FGR animal experiments from our group (104) and others (105, 106), which find no overt difference in pulmonary structure of FGR offspring. When we compare results between our preterm and term lamb cohorts, it is evident that the timing and duration of placental insufficiency is a critical determinant of lung dysfunction. We have recently examined the effects of early-onset placental insufficiency on lung structure and function, finding that lung cellular morphological changes are present (unpublished results). Accordingly, we propose that altered lung structural development is dependent on the timing of compromise, rather than the severity of growth restriction. Further, in early-onset FGR, the severity of fetal hypoxia has an inverse relationship with pulmonary surfactant production leading to decreased surfactant, a relationship not maintained in late-onset FGR (107). It is well accepted that without adequate surfactant, the newborn is at increased risk of pulmonary complications after birth, particularly when the infant is born preterm.

As discussed above, chronic hypoxia results in the fetal adaptive response of redistribution of cardiac output. MRI studies have confirmed that in late gestation of human fetuses, growth restriction is associated with an increased superior vena cava flow and, consequently, decreased pulmonary artery flow (108). This hemodynamic response is contributed by increased pulmonary vascular resistance (97). Increased pulmonary vascular resistance also reduces venous return to the left heart (109) and enhances right ventricular afterload. Combined ventricular output is thus maintained (108). Postnatally, FGR does not alter pulmonary blood flow during the transition to ex utero life, but left ventricular output is lower (110). Thus it is apparent that the hemodynamic adaptation to chronic hypoxia also has important implications for pulmonary vascular development, and accordingly, lung structure and function in FGR offspring.

A handful of studies have also examined fetal breathing movements in the developing fetus, and undertaken comparison in FGR versus appropriately grown fetuses. Fetal breathing movements are an important component of normal lung development, as they provide a stretch stimulus for growth throughout gestation (111). In FGR sheep, fetal breathing movements are significantly reduced in late gestation, although it is noted that not all experimental models of placental insufficiency show such changes (69). The cessation of fetal breathing movements in response to placental insufficiency is thought to occur by way of physiological response to reduce metabolic rate and thus conservation of oxygen, and is associated with disrupted alveolarization (112).

Deficits in pulmonary development subsequent to placental compromise are not confined to lung alveolar morphology. There is a growing understanding of the link between poor alveolar development and poor lung vascular development (113–115), called the *Vascular Hypothesis*. Growth restriction, induced via hyperthermia in pregnant sheep, impairs both lung alveolar and vascular development in the developing fetus (116). In complimentary experiments, lung alveolar cells isolated from the same growth-restricted fetuses demonstrate reduced cell growth, migration and branching, which are key components of normal lung development (116). These findings are confirmed *in vivo* in which growth-restricted offspring demonstrate diminished pulmonary vascular function and density, together with decreased pulmonary alveolarization (116, 117). Abnormal pulmonary vascular development in growth restricted fetuses is likely to be a key mechanism increasing the risk of BPD, pulmonary hypertension and life-long reduction in respiratory capacity, such as seen in chronic obstructive disease (116).

Impact

BPD is a chronic lung disease characterized by arrested airway and parenchymal development and resulting in long-term respiratory complications, with a high susceptibility in preterm and growth restricted infants. BPD is a multifactorial condition, however it is primarily thought to result from chronic ventilation-induced injury in preterm infants, contributed by lung exposure to excess oxygen and inflammation. FGR is an independent risk factor for BPD in human infants (118–120). Being born subsequent to placental insufficiency and growth restricted is associated with a 3.6-fold higher risk of developing BPD than age-matched control infants (120), despite FGR infants having similar RDS rates as appropriately-grown counterparts. Lio et al. (121) have also recently shown that FGR infants with placental dysfunction have a 6-fold increased risk of developing BPD compared to low birth weight/ SGA infants. Further, they noted that birth weight *per se* and not ventilation duration, or other neonatal morbidities, contributed to the presence of BPD.

Maternal vascular unit deficiency, a marker for pre-eclampsia, is a common placental pathology associated with FGR, and it has also been shown that maternal vascular unit dysfunction doubles the risk of BPD in preterm human infants (118). Thus, human and animal data strongly support that the foundations of postnatal lung deficits and BPD are laid down *in utero* in FGR infants with placental insufficiency, and that vascular pathology is likely to be a contributing factor.

Neonatal pulmonary hypertension is highly associated with decreasing gestational age and low birth weight, and is a common complication of BPD (96). Pulmonary hypertension is characterized by hypoxemia of the newborn and right-to left shunting through the ductus arteriosus, due to maintenance of high pressures within the pulmonary circulation. Accordingly, neonatal pulmonary hypertension occurs via a failure of structural cardiovascular remodeling after birth, and is likely developmentally programmed *in utero* (122). In post mortem tissue analysis it is shown that newborns with pulmonary hypertension displayed reduced pulmonary vascular surface area with increased muscularization of distal pulmonary vasculature (123). These data suggest a strong association with FGR induced by vascular remodeling in chronically hypoxic fetuses, resulting in impaired control of vascular tone within the pulmonary circulation after birth. Altered pulmonary vascular composition has been more closely examined in growth restricted rats, demonstrating increased pulmonary vasoconstriction caused by local endothelial dysfunction and excessive collagen and reduced elastin in the pulmonary vasculature (124). Animal models of FGR also provide strong evidence that the hallmarks of pulmonary hypertension are already present in the growth-restricted fetus and offspring soon after birth. Our group and others have shown vascular changes, including decreased vascular density and dysfunction in fetal sheep (116) and in 2-h-old lambs (110). Thus, even prior to birth, FGR is associated with pulmonary hypertension.

The long-term effects of low birth weight have been examined in adult offspring conceived during the 1940s Dutch famine, who show an increased risk of obstructive airway disease (89). Further analysis of this cohort determined that neither serum immunoglobulin E concentration nor mean lung volumes were different (125). The authors speculate that bronchial reactivity must be the cause of the airway disease following growth restriction.

Neurological Morbidity

Clinical Features

FGR is strongly linked to suboptimal brain development, and long-term neurological dysfunctions in motor ability, cognition and learning, and behavior. We have recently reviewed the consequences of placental insufficiency and FGR on the developing brain (28), and describe that the age of onset and severity of FGR, together with gestational age at birth, play important modulatory roles in altered brain structure and function. The first indication of structural anomalies of the FGR brain can be derived from magnetic resonance imaging (MRI) during fetal development. MRI of the fetal brain during development demonstrates reduced brain volume, and altered cortical folding and brain morphology in FGR fetuses (126, 127). Arthurs et al. (128) showed lower diffusion weighted imaging values in parts of the brain in severe FGR fetuses as compared to normal age-matched controls, which were suggestive of an abnormal maturational profile. Postnatally, at term-equivalent age, MRI detects reduced intracranial volume, particularly contributed by decreased cortical gray matter volume in FGR infants (129), and altered developmental profile of white matter myelination (130), the hippocampus (131) and the basal ganglia (132) of growth-restricted infants, compared to appropriately grown infants. Functional MRI is also an upcoming tool to study whole brain functional networks in newborn infants for the assessment of altered organization and prediction of long-term neurodevelopment (133). Diffusion tensor imaging (DTI) and connectivity-based analysis of the FGR brain in the neonatal period is also being increasingly investigated (134).

MRI has the ability to detect even relatively small volume, structural, and organizational differences within the brain of FGR and appropriately-grown infants (135) but MRI capability and expertise in analysis is not readily available at all birth centers. In contrast, neonatal cranial US is widely used, but shows less sensitivity for detection of these subtle, but important neurological changes associated with neuropathology in FGR infants (136). Cranial ultrasound is frequently used as an assessment tool in premature infants, and term infants with severe FGR, to identify significant neuropathology in the neonatal period. There remains uncertainty as to whether cranial ultrasound can adequately detect neuropathology associated with FGR when compared to age-matched appropriately grown preterm infants (135, 136). Certainly in older preterm and term FGR infants, the benefit of routine cranial ultrasound screening in the neonatal period is questionable (137). We did not find evidence of altered cerebral ventricular volume using ultrasound imaging in FGR infants <10th centile, however we did observe a correlation between increasing ventricular volume and a decrease in functional motor scores (138). Cruz-Martinez et al. (139) have suggested that FGR infants with signs of middle cerebral artery and other Doppler abnormalities (indicative of significant brain sparing) are more likely to have neuropathology that can be detected on neonatal cranial ultrasound. This is interesting, as it further supports that the term *brain sparing* is a misnomer, and while it represents an appropriate survival response in the fetus, it is actually associated with worsening fetal condition and greater brain injury (28).

FGR infants frequently have a reduced head circumference compared to age-matched appropriately-grown infants, which is likely due to reduced brain volume (129), and reduced brain volume persists to 12 months of age (140). Cerebellar and hippocampal volumes may also be reduced (130). Brain myelination and connectivity have been shown to be adversely affected in FGR infants in the first 12 months of life, representative of white matter injury (141). Diffusion MRI of the human brain shows that the overall neuronal network complexity and connectivity of the FGR brain is reduced, with reduced global and local axonal circuits (142). Long-range cortical-basal ganglia (thalamocortical) connections are decreased in children born preterm with FGR, compared to children born preterm

but appropriately-grown (142), indicating that brain connectivity is significantly worse in children who were FGR compared to children who were preterm but well-grown. Deficits in brain connectivity correlate with neurobehavioral impairments including hyperactivity and poor cognition at school in children who were born FGR (143).

Neonatal functional assessment may detect early problems with neurological processing and behavior in infants who were born growth restricted. Tolsa et al. (129) showed that FGR newborns had specific alterations of brain structure as studied by volumetric MRI at preterm and term age, with reduced cortical gray matter volume correlating with deficits in attention and responsivity at term-equivalent age. General movement assessments (Prechtl movements) provide an early motor analysis, wherein abnormalities are predictive for cerebral palsy, and general movements may be adversely affected in some FGR infants (144). Similarly, electroencephalography performed early in the neonatal period has been shown to be affected and may correlate with adverse neurodevelopment in studies of FGR infants (145, 146). There is however limited data on early detection of functional deficits in growth-restricted infants, reflecting challenges in detecting delayed neurodevelopment in the neonatal period.

Pathophysiology

It is now well established that the traditional brain sparing physiology does not necessarily mean normal cerebral development *in utero* (28). In fact, fetuses with the most severe brain sparing are at the highest risk of adverse neurodevelopment in childhood. Prenatal loss of vasoreactivity in FGR has been suggested as a mechanism for poor outcomes, in which fetuses who do not adjust their cerebral circulatory control in response to hypoxic challenge may be more at risk of impaired cerebrovascular regulation (147). There are also reports of preferential perfusion and cerebral redistribution of brain blood flow in FGR fetuses, leading to some brain regions being at higher risk of injury (148). This is supported by work in fetal sheep to demonstrate that FGR is associated with regional cerebral blood flow redistribution, with the most notable differences between FGR and appropriately grown fetuses seen in the cerebral cortex and periventricular white matter (21).

Cerebral blood flow frequently continues to be abnormal for the first few days after birth in FGR human infants, but whether this puts infants at an increased risk of acute brain injury is not known. It has been reported the cerebral blood flow remains elevated after birth in FGR infants (149), even when the neonate is no longer exposed to a hypoxic environment and is no longer in need of a compensatory change in cardiac output. Postnatally, elevated cerebral blood flow might potentiate hyperoxia and oxidative stress within the fragile brain, which could also contribute to further neurological damage.

Animal models of chronic hypoxia and growth restriction have helped us to understand the development of neuropathology associated with placental insufficiency and FGR (28, 150–152). Adverse effects on brain gray matter development, white matter, and cerebellum have been described both in sheep, rabbit and rat models of FGR (153–155). In fetal sheep, we showed that early-onset placental dysfunction is associated with more widespread and severe white matter brain injury and neuroinflammation compared with late-onset, however both early- and late-onset FGR demonstrate complex patterns of gray and white matter neuropathology (154). Animal studies also show that the severity of brain injury, and the resultant neurodevelopment deficits, depends on the extent and severity of brain involvement in FGR (156). Hypomyelination and delayed myelination due to oligodendrocyte maturational deficits have been identified as possible mechanisms causing the white matter injury seen in FGR infants (157). Deficits in neuronal connectivity have also been described in animal models (158). Our group, and others, has observed that deficits in various components of the neurovascular unit play a significant role in the brain injury seen in animal models of FGR (159). Prematurity is a confounder in human FGR, but studies in FGR animals allow the separation of growth restriction and preterm birth. The individual contributions of preterm birth and/or neonatal ventilation of the FGR newborn on the progression of brain injury are now being examined (160, 161). These studies have determined that preterm birth and ventilation synergistically predispose the vulnerable FGR brain to neuropathology.

Impact

FGR infants are at increased risk of adverse neurodevelopmental outcomes in childhood. Neurological morbidities related to motor deficits, including cerebral palsy, behavioral issues, and cognitive impairment is significantly increased in young children and adolescents who were diagnosed as growth restricted at birth (28, 162–164). The risk of cerebral palsy is 30-fold greater in FGR infants, compared to those that are well grown (165), and increases with worsening growth restriction. Overall, >40% of children who have cerebral palsy had a low birth weight; that is, they were growth restricted, born preterm, or both (166). This is important, as FGR and preterm birth are frequent co-morbidities. In addition to motor deficits, preterm FGR infants followed-up at 1, 2, and 3 years of age showed deficits in cognition and behavioral outcomes compared to preterm age-matched appropriately-grown infants (167). Further, a longitudinal study observing FGR offspring with evidence of brain sparing from birth to middle school age (9–10 years old) found a complex set of neurodevelopmental deficits, such as a significant reduction in IQ, compared to age-matched appropriately-grown children (168). Multiple follow-up studies of FGR infants into school age describe diminished gross and fine motor skills, cognition, memory, and academic ability, as well as neuropsychological dysfunctions encompassing poor attention, hyperactivity and altered mood (143, 169–171). FGR infants born preterm and those with fetal circulatory redistribution are at the greatest risk for the worst outcomes (172). These adverse outcomes can continue into adolescence and young adulthood (173). It is apparent that determining the neurodevelopmental consequences of FGR is complicated by the severity of FGR, early- or late-onset, and the gestational age at delivery (28). However, in both early- and late-onset FGR, the presence of

cardiovascular redistribution and brain-sparing is associated with abnormal neurodevelopmental outcomes (28).

Interventions for Improved Outcomes

Management of pregnancies complicated by FGR represents a balance between antenatal compromise, often with worsening chronic hypoxia that contributes to subpotimal organ development, and the risks associated with preterm delivery and postnatal intensive care, which may also contribute to morbidities. In high-income countries, about half of fetuses with moderate- to severe-growth restriction are detected antenatally and are therefore amenable to treatment during pregnancy, but it remains that nearly 40% of infants born at the 3rd centile for weight are not detected *in utero* (44). With this in mind, both antenatal and postnatal therapies must be considered. Currently, no specific treatment is available for FGR. Potential treatments should target maldevelopment of multiple organs, various injurious pathways, cell types, and structural deficits that manifest over different developmental stages. Here we will provide an overview of the current state of understanding for a handful of treatments for FGR (**Figure 3**).

Antenatal

Antenatal treatments are principally aimed at improving placental function and thereby increasing fetal growth *in utero*. To date, the best studied of these has been sildenafil citrate. Sildenafil is a potent phosphodiesterase type 5 (PDE5) inhibitor that is an effective smooth muscle relaxant where the PDE5 enzyme is present in an organ or tissue, as is the case for the human placenta (174). The effects of sildenafil on smooth muscle are mediated via an enhanced and prolonged nitric oxide release leading to vasodilatation. Both *in vitro* and *in vivo* studies demonstrate that sildenafil vasodilates human myometrium vessels from normal (175, 176) and growth restricted placenta. Most experimental studies to date support that sildenafil increases fetal weight in compromised rat, sheep and human pregnancies (177). In contrast, we have shown that antenatal sildenafil administration to pregnant sheep with placental insufficiency decreases fetal weight and worsens fetal hypoxia (178). Although initial preclinical evidence for the multinational STRIDER trial suggested improved outcomes for FGR infants, this trial has now been aborted due to unexpected baby deaths (179), leading to a call for increased preclinical studies underpinning clinical trials (180), and improved understanding of the effects of sildenafil on the fetus given that it crosses the placenta (181). The longer acting tadalafil remains an active clinical experimental treatment of interest as an antenatal therapy for FGR and, given that tadalafil does not cross placenta (174), it may be more favorable as a targeted placental treatment.

The EVERREST Project is also investigating a targeted approach to improve placental function in pregnancies complicated by FGR using gene therapy to inject vascular endothelial growth factor (VEGF) into uterine arteries (182). VEGF is known for its role in inducing angiogenesis and in the EVERREST Project it is hypothesized that application of adenovirus VEGF in, or near placental arteries will induce a local and acute increase in VEGF expression, and subsequent angiogenesis of the placental vasculature. Preclinical studies have shown promise with improved blood flow (183) and fetal weight gain (184) in animal models of growth restriction, resulting from the improved vascularization of the placenta. The clinical trial is ongoing.

A recent large animal (sheep) study examined intra-amniotic administration of the growth-promoting protein insulin-like growth factor-1 (IGF-1) (16). This work showed that increasing the bioavailability of IGF-1 in pregnancies complicated by placental insufficiency and FGR improved birth weight in female lambs, but not males, and modified postnatal catch-up growth in both females and males. Intrauterine IGF-1 also mediated expression of key somatotrophic and metabolic genes, indicative that antenatal treatment could be utilized to positively affect postnatal growth and wellbeing.

A number of antenatal treatments have been explored preclinically that aim to restore fetal oxidative tone via maternal antioxidant administration, using agents such as allopurinol, melatonin and vitamin C (75, 79, 185). Antioxidant treatment has principally targeted improved cardiovascular and neurological outcomes in growth-restricted offspring. To date, melatonin has been the most widely studied, given melatonin's established safety profile, ease of administration, and strong antioxidant benefits. In sheep, we have shown that maternal melatonin administration to ewes carrying a growth restricted fetus results in a significant improvement in vascular function and reduced arterial stiffness, two vital pathologies evident in FGR offspring, which predispose to cardiovascular disease (75). Melatonin administration also resulted in improved cardiac function in the right ventricle. Further, this study showed that maternal melatonin improved fetal oxygenation and increased birth weight (75), however other ovine studies show either no improvement in birth weight (186) or exacerbation of growth restriction with melatonin (187). In cultured human umbilical vein endothelial cells (HUVECs), melatonin improves vascular endothelial integrity, likely via combined anti-oxidant and anti-inflammatory mechanisms (188). Exposure to antenatal melatonin does not reverse alveolar simplification in FGR newborn lambs (102), but does improve pulmonary vascular structure and function (189), and pulmonary tone may be maintained long term via alteration to receptor populations (190). As our understanding of perturbations to lung growth in FGR offspring continues to be explored, so too does the opportunity for targeting novel pathways. For example, recent work has shown that NPY is down regulated in FGR, where NPY is a sympathetic neurotransmitter that is critical for normal lung growth (191).

The effects of maternal melatonin administration on brain development have also been examined. Antenatal melatonin crosses the placental and the blood brain barrier, and melatonin is a strong antioxidant and also demonstrates anti-inflammatory benefits in the developing brain (186, 192–194). In pregnancies complicated by placental insufficiency and FGR, maternal melatonin improves white matter brain development via increased myelination and decreased axonopathy in the fetal brain, and subsequently, neurobehavior of FGR+MLT lambs is significantly improved after birth (186). Melatonin has also been shown to have beneficial effects on cerebral vasculature

by preventing FGR-related apoptosis and disruption of blood brain barrier instability via improved vascular interactions with astrocytes and pericytes (195). Antenatal melatonin has been examined in pilot studies to treat FGR (186) and preeclampsia (188) with results supporting that melatonin is an effective anti-oxidant that is safe for the mother and baby, and may extend pregnancy (196).

Emerging evidence supports that the glycoprotein lactoferrin shows potential as an antenatal treatment for pregnancies complicated by FGR, particularly for the developing brain (197). Lactoferrin is a glycoprotein that demonstrates strong antioxidant, anti-inflammatory and anti-microbial effects—important factors that could mediate neuroprotective benefits. In rats, lactoferrin supplementation during pregnancy shows positive benefits for dexamethasone-induced fetal growth restriction (197). Maternal lactoferrin significantly increased birth weight of control rat pups, and FGR offspring exposed to lactoferrin showed a normalized weight at postnatal day 21. Lactoferrin supplementation also improved brain hippocampal structure and stimulated brain derived neurotrophic factor (BDNF) (197), important observations in light of the neuropathology associated with human FGR. Nutritional supplementation (glucose, amino acids and electrolytes) into the amniotic sac of FGR rabbits has also recently been explored, with some promising results suggesting that survival rate for FGR offspring was improved with treatment, although birth weight and cardiac function deficits were not improved (198).

Postnatal

As mentioned above, nearly 40% of human infants with severe FGR are not detected antenatally (44), and therefore not amenable to antenatal treatments. In light of this we must continue to investigate therapies to improve multi-organ dysfunctions in growth restricted infants. We have highlighted in this paper that deficits in cardiovascular, pulmonary and cerebral development are already present at birth in FGR infants, principally caused by chronic hypoxia *in utero*. Therefore any potential postnatal therapy would aim to be reparative and to prevent progression of ongoing multi-organ damage.

Lactoferrin shows great potential as a postnatal therapy, in addition to positive effects antenatally. Lactoferrin is highly abundant in human colostrum and milk, and it reaches the brain after oral administration (197, 199). In this regard, breastfed infants show higher total anti-oxidant capacity and a lower oxidative stress index compared to non-breastfed infants. Importantly, randomized controlled trials with nutritional lactoferrin supplementation in premature neonates demonstrate a promising reduction in late onset sepsis and necrotizing enterocolitis (200). Lactoferrin supplementation during lactation is protective for neonatal rats exposed to either hypoxia-ischemia or lipopolysaccharide-induced systemic inflammation (201, 202). Analysis of the neonatal rat brain using a combination of advanced MRI analysis and histology demonstrates that gray and white matter microstructure is normalized with lactoferrin supplementation, myelination is protected and measures of axonal integrity and brain organization are restored in rats with lactoferrin supplementation (201). Early environment enrichment or postnatal stimulation has also been shown to have some benefits in brain connectivity in a rabbit model of FGR (203). While there are no current published studies on stem cell therapy for FGR related brain injury, our lab and others are working on testing their applications in FGR and other pregnancy complications (204). The application of postnatal therapies to improve multi-organ deficits associated with FGR should remain a foremost preclinical research area.

CONCLUSIONS

Understanding the pathophysiological mechanisms that underlie neonatal morbidities that are particularly associated with FGR provide the fundamental basis for improving short- and long-term outcomes in growth restricted offspring. It is clear that placental compromise and chronic fetal hypoxia program the fetus for suboptimal growth and development, with fetal cardiovascular dysfunctions and altered organ development already apparent in the FGR fetus during pregnancy. The timing of the onset of placental insufficiency, the severity of growth restriction, the degree of cardiovascular adaptation, and gestational age at birth are all critical factors that modify outcome for FGR infants. In the neonatal period, FGR infants demonstrate early evidence of cardiac, vascular, pulmonary, neurological and other deficits, which can lead to long durations in neonatal intensive care, and long-term health problems. Improved antenatal detection, and both antenatal and postnatal therapies that target the key pathophysiological mechanisms underlying altered multi organ structure and function must be considered critical research areas.

AUTHOR CONTRIBUTIONS

AM and BA critically researched literature, co-wrote the first draft of the manuscript and approved the final version. MC-M, GJ, GP, and SM reviewed the manuscript, contributed to various sections and approved the final version.

ACKNOWLEDGMENTS

The authors wish to thank their funding supports: National Health and Medical Research Council (NH&MRC) project grant, Cerebral Palsy Alliance grant, Royal Australasian College of Physicians Foundation Fellowship (AM); NH&MRC Research Fellowships (GP and SM), National Heart Foundation of Australia (GP), Australian Research Council Future Fellowship (SM) and the Victorian Government's Operational Infrastructure Support Program.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

SYSTEMATIC REVIEW
published: 08 February 2019
doi: 10.3389/fendo.2019.00058

Intrauterine Growth Restriction and Patent Ductus Arteriosus in Very and Extremely Preterm Infants: A Systematic Review and Meta-Analysis

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OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *26 September 2018*
Accepted: *22 January 2019*
Published: *08 February 2019*

Citation:
Villamor-Martinez E, Kilani MA, Degraeuwe PL, Clyman RI and Villamor E (2019) Intrauterine Growth Restriction and Patent Ductus Arteriosus in Very and Extremely Preterm Infants: A Systematic Review and Meta-Analysis. Front. Endocrinol. 10:58. doi: 10.3389/fendo.2019.00058

It is generally accepted that intrauterine growth restriction (IUGR) increases morbidity and mortality among very preterm neonates. However, evidence is hampered by the widespread practice of using the terms small for gestational age (SGA) and IUGR as synonyms. We conducted a systematic review of studies reporting on the association between IUGR/SGA and patent ductus arteriosus (PDA). PubMed/MEDLINE and EMBASE databases were searched. Of 993 studies reviewed, 47 (50,790 infants) were included. Studies were combined using a random effects model and sources of heterogeneity were determined by subgroup and meta-regression analyses. Meta-analysis of all included studies showed a significantly reduced risk of PDA in the SGA/IUGR group with an odds ratio (OR) of 0.82, and a 95% confidence interval (CI) of 0.70 to 0.96 ($p = 0.015$). Of the 47 studies, only 7 used a definition for growth restriction that went beyond birth weight (BW) for gestational age (GA). When pooled, meta-analysis could not demonstrate a significant effect size (OR 1.31, 95% CI 0.75 to 2.27, $p = 0.343$). Moreover, the significantly reduced risk of PDA was found in the 25 studies defining SGA as BW <10th percentile (OR 0.81, 95% CI 0.66 to 0.98, $p = 0.032$), but not in the 6 studies defining SGA as BW <3rd (OR 1.09, 95% CI 0.70 to 1.71, $p = 0.694$), or in the 27 studies using a more refined definition of PDA (i.e., hemodynamically significant PDA or PDA requiring treatment, OR 0.87, 95% CI 0.72 to 1.04, $p = 0.133$). In addition, we found that GA was significantly higher in the SGA/IUGR group (18 studies, mean difference 0.63 weeks, 95% CI 0.24 to 1.03, $p = 0.002$). Meta-regression analysis confirmed the correlation between this difference in GA and PDA risk. In summary, we observed marked heterogeneity across studies in the definition of growth restriction and PDA, and we found differences between the control and growth-restricted groups in relevant baseline characteristics, such as GA. Therefore, our meta-analysis could not provide conclusive evidence on the association between growth restriction and PDA.

Keywords: small for gestational age, growth restriction, patent ductus arteriosus, very preterm infant, meta-analysis, meta-regression

INTRODUCTION

The ductus arteriosus (DA) of very preterm infants is less likely to close spontaneously as part of the transition to extrauterine life and, consequently, the incidence of patent DA (PDA) is inversely related to gestational age (GA) at birth (1–4). Intrauterine or fetal growth restriction (IUGR/FGR) is commonly recognized as an additional major risk factor for mortality and morbidity in very preterm infants (5–11). One of the conditions that IUGR has been associated with is PDA (12–14), but the evidence supporting this association is scarce and has not been systematically reviewed. Some studies even suggest that IUGR may protect against PDA (15–18).

A common problem of studies assessing the potential association of growth restriction with adverse neonatal outcomes is that they do not differentiate between small for gestational age (SGA) and IUGR, even though the two terms are not synonymous (7–10). SGA is a statistical definition based on birth weight (BW), with the 10th percentile as the most commonly used threshold. The term SGA differs from IUGR principally because it also encompasses constitutionally small but healthy infants at lower risk of abnormal perinatal outcome. On the other hand, growth restricted infants who have a BW above the 10th percentile may be falsely classified as normally grown (6–10).

The most common cause of IUGR is placental insufficiency leading to fetal hypoxia and undernutrition (19). Whether the normal development of the DA is affected by these pathological conditions remains largely unknown. Experimental animal studies showed that reactivity of DA is impaired by exposure to chronic fetal hypoxia (20, 21). King et al. showed histological evidence of accelerated DA maturation in very preterm infants exposed to chronic intrauterine stress, leading to the hypothesis that this may have resulted in earlier postnatal DA closure (22). In contrast, Ibara et al. described alterations in the DA of preterm infants with IUGR (23). These include fragmentation, coagulation and necrosis of the internal elastic lamina, as well as hemorrhage with necrosis and loosening of elastic fibers and muscles in the tunica media (23). These ductal changes may explain why hemodynamically significant PDA (hsPDA) has been reported to occur more frequently and at an earlier postnatal age in very preterm infants with IUGR (12, 13).

We aimed to carry out a systematic review of observational studies reporting on the association between IUGR/SGA and PDA. We paid particular attention to how the criteria used to define growth restriction and PDA affected the potential association between the two conditions. We also analyzed the role of potential confounders, such as GA and rate of respiratory distress syndrome (RDS).

METHODS

The methodology of this study is based on that of earlier studies of our group on chorioamnionitis and various morbidities (24–26) and PDA and platelet counts (27). The study is reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (28). The PRISMA checklist is included in the **Supplementary Material**. We developed a protocol a priori defining the objectives, methods, inclusion criteria and approach to assessing study quality.

Data Sources and Search Strategy

We performed a comprehensive systematic literature search using the PubMed/MEDLINE and EMBASE databases. The first search was performed on October 1st, 2016. Automated alerts were used during the elaboration of the review to maintain the search up to date. The search strategy was as follows for PubMED: (PDA OR ductus arteriosus) AND (preterm OR premature) AND (IUGR OR growth restriction OR growth retardation OR restricted growth OR fetal growth OR fetus growth OR reduced growth OR prenatal growth OR placental dysfunction OR placental insufficiency OR chronic hypoxia OR chronic hypoxemia OR small for gestational age OR small for date OR SGA). We used a similar strategy for EMBASE. There were no language preferences set. Reference lists of relevant primary and review articles were searched for additional studies. The "cited by" function in Web of Science and Google Scholar was also used to expand the search parameters and ensure that all relevant studies were found.

Study Selection

We included studies which reported on a growth restricted (SGA, IUGR, FGR) group and a comparison group, studied (very and extremely) preterm infants, and reported primary data that could be used to measure the association of SGA/IUGR and PDA. To assess relevance, two reviewers (MAK, PLD) screened the results of the searches and applied inclusion criteria using a structured form. Disagreements were resolved through discussion or in consultation with a third reviewer (EV).

Data Extraction

A predetermined designed data extraction form was used. Data was extracted from relevant studies by two investigators (MAK, PLD). Accuracy and completeness of the data extraction was then assessed by two other investigators (EVM, EV). Data extracted from each study included citation information, country where research was conducted, language of publication, study design, objectives, inclusion/exclusion criteria, definitions of SGA/IUGR and PDA, patient characteristics [including GA and birth weight (BW)], and results (including raw numbers and adjusted analyses on SGA/IUGR and PDA where available). When studies assessed PDA at several time points, we used the PDA incidence at the last time of assessment for data analysis.

Quality Assessment

We assessed study quality using the Newcastle-Ottawa Scale for assessing the quality of non-randomized studies in meta-analyses. This scale allocates points for quality in the domains of selection (0–4 points), comparability (0–2 points), and outcome/exposure (0–3 points), for a total of 0–9 points. The process was carried out by two reviewers independently (EVM and EV). Discrepancies were resolved through discussion.

Statistical Analysis

Studies were pooled and analyzed using COMPREHENSIVE META-ANALYSIS V 3.0 software (CMA, RRID:SCR_012779, Biostat Inc., Englewood, NJ, USA). For dichotomous outcomes, the odds ratio (OR) with 95% confidence interval (CI) was calculated from the data provided in the studies. For continuous outcomes, the mean difference (MD) with 95% CI was calculated. When studies reported continuous variables as median and range or interquartile range, the mean and standard deviation were estimated using the method of Wan et al. (29).

Summary statistics were calculated with a random-effects model because of anticipated heterogeneity. This model accounts for variability between and within studies. Subgroup analyses were used to analyze sources of heterogeneity. They were based on the mixed-effects model (30). In this model, a random effect model is used to pool studies within each subgroup and a fixed effect model is used to combine the subgroups and generate the summary effect. We assumed a common among-study variance component (tau-squared) across subgroups.

We determined a priori that we would create subgroups for SGA/IUGR definition, PDA definition, and for studies which only included extremely preterm infants (GA <28 weeks). We also decided to carry out meta-analyses of the following covariates in the SGA and control groups: GA, BW, rate of RDS, and rate of antenatal corticosteroids (ACS). Statistical heterogeneity was assessed using Cochran's Q statistic and I^2 statistic, which is a derivative from Q and demonstrates the chance of total variation resulting from heterogeneity beyond chance (30). A univariate random-effects meta-regression (method of moments) was used to investigate the role of GA, rate of RDS, and rate of ACS in explaining differences in effect sizes among studies (30). Meta-regression was also used to compare subgroups. Publication bias was evaluated by using Egger's regression test and through visual inspection of funnel plots. We considered a probability value of less than 0.05 (0.10 for heterogeneity) as statistically significant.

RESULTS

Included Studies

We identified 993 potentially relevant studies from which 47 (50,790 patients, 7,860 SGA/IUGR cases, 17,300 PDA cases) met the inclusion criteria (5, 12, 13, 15–18, 31–70). The PRISMA search diagram is depicted in **Supplementary Figure 1** and the main characteristics of the included studies are shown in **Supplementary Table 1**.

While all studies provided data to measure the association between SGA/IUGR and PDA, only 3 of the studies were primarily designed to assess this association (13, 32, 69). Twenty-three studies reported on risk factors for PDA, and IUGR/SGA was one of the risk factors considered (**Supplementary Table 1**). Twenty-one studies examined the outcomes of IUGR/SGA infants, and PDA was one of the outcomes studied (**Supplementary Table 1**).

Forty studies defined growth restriction based on BW below a determined percentile for GA (**Figure 1**). From these studies, 25 used the 10th percentile, 2 studies used the 5th percentile, and 6 studies used the 3rd percentile (or −2 standard deviations) (**Figure 1**). Seven studies did not specify which criteria or percentile was used to define growth restriction (**Figure 1**). One study used an ultrasound estimated fetal weight chart (48). Six studies used customized BW charts, which adjusted for factors such as sex, ethnicity, socioeconomic status, or parity (15, 17, 35, 50, 57, 63). Thirty-three studies used population-based BW charts (**Supplementary Table 1**). Eighteen of these 33 studies used sex-specific charts, and 15 used charts that did not differentiate by sex.

Seven studies used a definition of growth restriction that went beyond the use of BW for GA and that included fetal assessment. Of these, 2 studies used a definition of deviation of fetal growth (13, 61), and in 5 studies fetal growth restriction was defined through the presence of an abnormal Doppler result (absent end-diastolic flow in the umbilical artery) (12, 41, 44, 46, 47).

The assessment of PDA was based solely on clinical criteria in one study (31), whereas the other studies used heart ultrasound or ultrasound combined with clinical criteria. Thirteen studies defined PDA as significant or hemodynamically significant PDA, and 14 studies defined PDA based on the necessity of treatment (**Figure 2** and **Supplementary Table 1**). Two studies defined all ductal shunts, including the small ones, as PDA (**Supplementary Table 1**). In 7 studies a definition or definition criteria for PDA was not specified (**Supplementary Table 1**).

Quality Assessment

The quality of each study was assessed using the Newcastle-Ottawa Scale and is summarized in **Supplementary Table 2**. Twelve studies scored 6 points (out of 9), 24 studies scored 7 points, and 11 studies scored 8 points. Studies were downgraded in quality mostly for not adjusting/matching for confounders ($k = 44$), for not clearly defining SGA/IUGR ($k = 7$), and for not clearly defining PDA ($k = 9$).

Meta-Analysis Results

As shown in **Figure 1**, meta-analysis of all 47 included studies found a significant negative association between being SGA/IUGR and developing PDA (OR 0.82, 95% CI 0.70–0.96). Neither visual inspection of the funnel plot (**Supplementary Figure 2**) nor Egger's regression test ($p = 0.079$) suggested publication bias. The association between growth restriction and PDA remained negative and significant when only evaluating studies which defined SGA through BW for GA (40 studies, OR 0.79, 95% CI 0.67–0.93). We further divided this group of studies according to the cut-off percentile. In the meta-analyses of studies using the 10th percentile (25 studies, OR 0.81, 95% CI 0.66–0.98) and studies where the percentile was not reported (7 studies, OR 0.55, 95% CI 0.36–0.86) the significant negative association between SGA and PDA was maintained (**Figure 1**). In contrast, meta-analyses of studies using the 5th percentile (2 studies, OR 0.63, 95% CI 0.26–1.52), and meta-analysis of studies using the 3rd percentile (6 studies, OR 1.09, 95% CI 0.70–1.71) could not demonstrate a significant association between being SGA and PDA (**Figure 1**). When examining the subgroup of studies that used a definition for IUGR that went beyond BW for GA (i.e., presence of abnormal

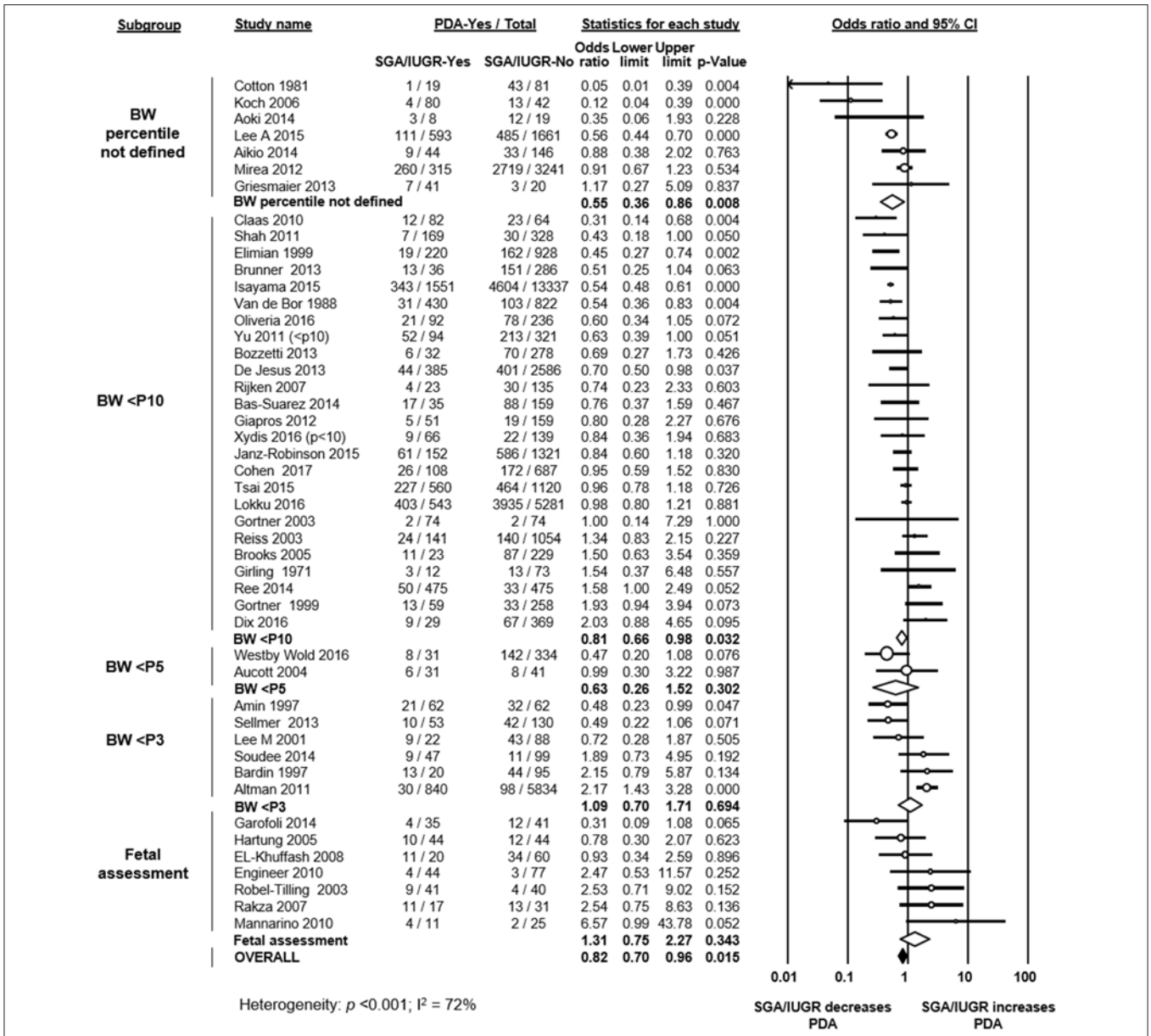

FIGURE 1 | Meta-analysis of the association of small for gestational age (SGA)/intrauterine growth restriction (IUGR) and patent ductus arteriosus (PDA). CI: confidence interval; BW: birth weight; <P10: BW lower than 10th percentile; <P5: BW lower than 5th percentile; <P3: BW lower than 3rd percentile.

Doppler or assessment of fetal growth), meta-analysis could not find a significant association between IUGR and PDA (7 studies, OR 1.31, 95% CI 0.75–2.27, **Figure 1**). When further subdividing by definition of IUGR, the association remained non-significant for the 5 studies using the abnormal Doppler criteria (OR 1.67, 95% CI 0.71–3.96) and for the 2 studies assessing fetal growth (OR 0.89, 95% CI 0.24–3.38).

To evaluate the role of PDA definition, we performed a further meta-analysis including only studies which defined PDA as hemodynamically significant or PDA requiring treatment. This meta-analysis could not find a significant association between SGA/IUGR and PDA in either subgroup (hsPDA: OR 0.92, 95% CI 0.71–1.20; PDA requiring treatment: OR 0.82, 95% CI 0.64–1.06), or when combining the two subgroups (OR 0.87, 95% CI 0.72–1.04, **Figure 2**).

To explore sources of heterogeneity, we carried out several additional meta-analyses of covariates, subgroup analyses and meta-regression analyses. Firstly, we examined the role of GA as a confounder in the association between SGA/IUGR and PDA. Six case-control and 13 cohort studies reported data on GA in the SGA/IUGR and the control group. Meta-analysis found that infants in the SGA/IUGR group were born significantly later (18 studies, MD 0.63 weeks, 95% CI 0.24 to 1.03, **Figure 3**) than infants in the control group. Although this effect was more

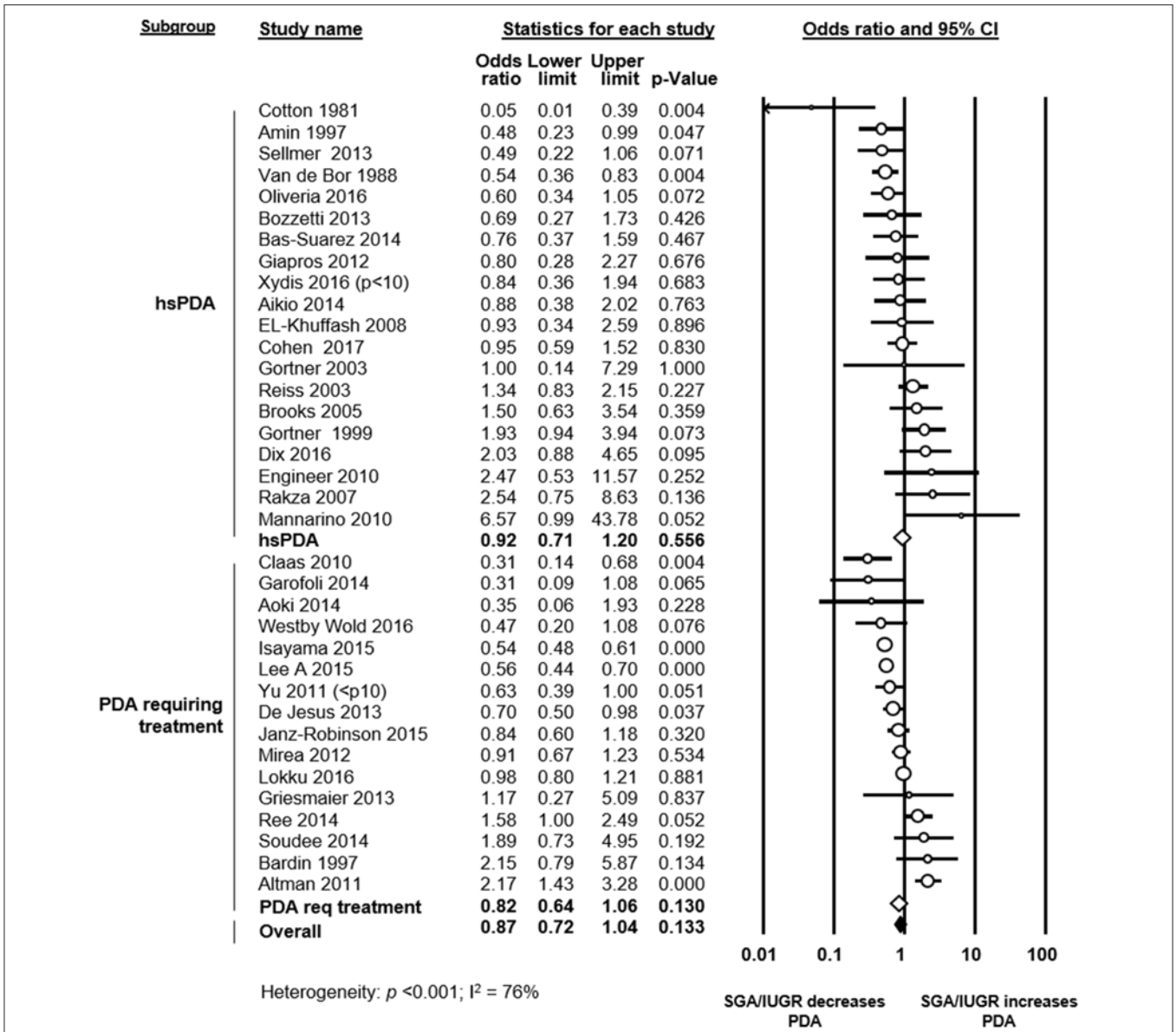

FIGURE 2 | Subgroup meta-analysis of studies using a definition of significant patent ductus arteriosus (PDA) (i.e., hemodynamically significant PDA or PDA requiring treatment). Analysis on the association of growth restriction and PDA. Req: requiring; CI: confidence interval; SGA: small for gestational age; IUGR: intrauterine growth restriction; hsPDA: hemodynamically significant PDA.

pronounced in cohort studies, it was also found in some case-control studies (**Figure 3**). The difference in GA between the SGA and the control group was particularly marked in the 3 studies (16, 33, 49) that only used BW as inclusion criterion. When these three studies were pooled, meta-analysis showed a MD in GA of 2.23 weeks (95% CI 1.66–2.79). Meta-analysis also found, as expected for a condition defined primarily using BW, that infants in the SGA group were significantly lighter at birth (18 studies, MD −379 g, 95% CI −452 to −306, **Table 1**). Both meta-analyses of GA and BW showed high statistical heterogeneity (**Table 1**).

We performed additional sensitivity analysis to investigate the possible influence of the differences in GA between the SGA/IUGR and the control group on the association between PDA and growth restriction. Meta-analysis of studies where infants with growth restriction were born more than 0.5 weeks later than control infants, found a protective effect of being growth restricted against PDA (**Table 2**). In contrast, meta-analysis of studies where growth-restricted infants were born <0.5 weeks later than control infants could not find a significant effect of growth restriction on PDA (**Table 2**). Meta-regression confirmed that the difference in effect size between these subgroups was statistically significant (p = 0.009, **Supplementary Figure 3**). When we grouped the studies according to the criteria of having or not having a

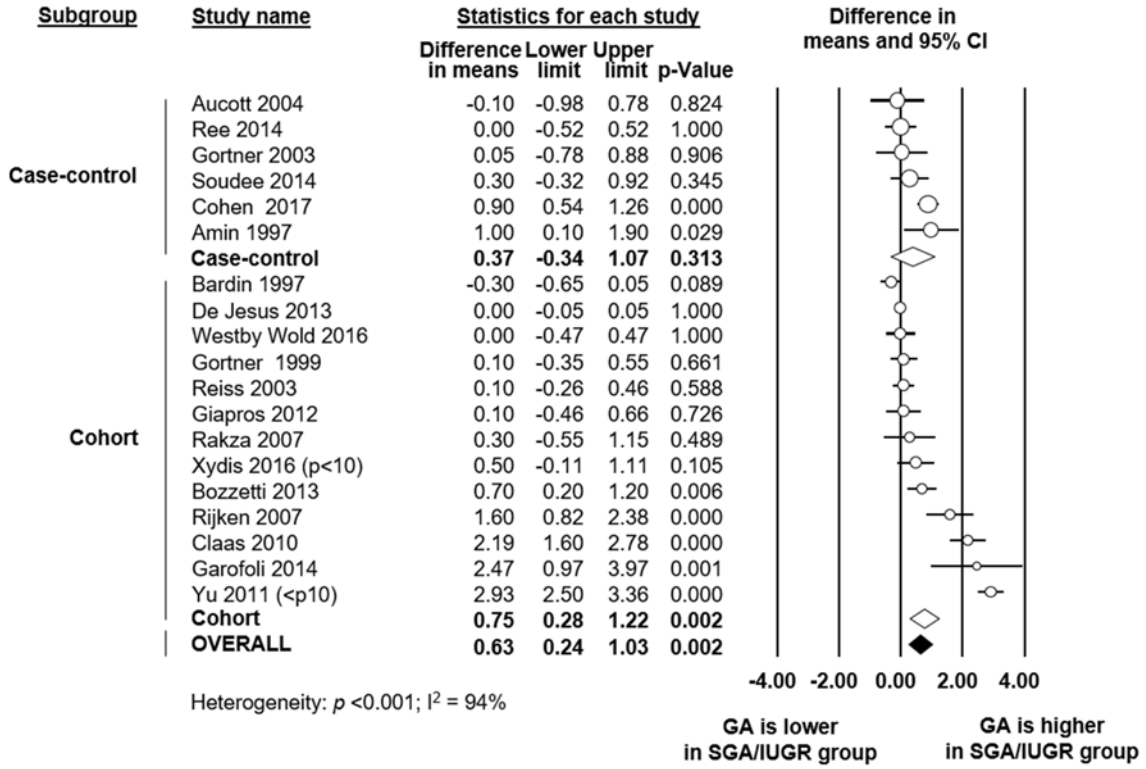

FIGURE 3 | Meta-analysis of mean difference in gestational age (GA) between growth restricted group and control. CI: confidence interval; SGA: small for gestational age; IUGR: intrauterine growth restriction.

TABLE 1 | Meta-analyses of confounding variables.

Meta-analysis	*k*	Effect size	95% CI	*p*	Heterogeneity	
					I^2	*p*
Gestational age (MD)	18	0.63 weeks	0.24 to 1.03	0.002	94%	<0.001
Birth weight (MD)	18	−379 g	−452 to −306	<0.001	97%	<0.001
Antenatal corticosteroids (OR)	17	1.18	0.94 to 1.49	0.159	59%	0.001
Respiratory distress syndrome (OR)	23	0.77	0.60 to 0.98	0.035	78%	<0.001

CI: confidence interval; k: number of studies; MD: mean difference (growth restricted group minus control group); OR: odds ratio.

statistically significant difference ($p < 0.05$) in GA between the SGA/IUGR and the control group, we found similarly that growth restriction was only a protective factor for PDA when growth-restricted infants were born significantly later than control infants (**Table 2**), and this difference between subgroups was also confirmed through meta-regression ($p = 0.008$, **Supplementary Figure 4**). In addition, meta-regression showed a significant linear correlation between MD in GA and risk of PDA (**Figure 4**). Sensitivity analysis of studies which only included infants with a GA <28 weeks or a <1,000 g BW found a significant association between SGA/IUGR and PDA (**Table 2**, **Supplementary Figure 5**).

In another subgroup analysis, we tested whether the studies that screened all infants for PDA (see **Supplementary Table 1**) showed a different effect size for the association between SGA/IUGR and PDA. As shown in **Table 2** and **Supplementary Figure 6**, universal screening for PDA did not significantly affect the OR of the association between SGA/IUGR and PDA (meta-regression $p = 0.990$). Finally, we performed additional meta-analyses to investigate whether the rate of use of ACS and the rate of RDS were different in the SGA/IUGR and the control group. As shown in **Table 1**, rate of ACS use was not significantly different but RDS rate was significantly reduced in the SGA/IUGR group. However, the significant negative association between SGA/IUGR and RDS was only observed for the subgroup of studies using the 10th percentile definition of SGA (OR 0.70, 95% CI 0.51–0.95). Meta-regression could not demonstrate a significant correlation between the effect size of the association between SGA/IUGR and PDA and the effect size of the association

TABLE 2 | Subgroup analyses.

Subgroup criteria	*k*	OR	95% CI	*p*	Heterogeneity	
					I^2	*p*
SGA/IUGR-group had a MD in GA <0.5 weeks compared to control	10	1.19	0.82–1.71	0.357	57%	0.014
SGA/IUGR-group had a MD in GA ≥0.5 weeks compared to control	8	0.60	0.41–0.89	0.010	16%	0.303
SGA/IUGR-group did not differ significantly in GA from control ($p \geq 0.05$)	11	1.15	0.81–1.63	0.432	52%	0.022
SGA/IUGR-group did differ significantly in GA from control ($p < 0.05$)	7	0.58	0.38–0.87	0.009	24%	0.244
All infants had GA ≤28 weeks or BW ≤1,000 g	28	0.81	0.67–0.97	0.020	55%	<0.001
All infants were screened for PDA	16	0.72	0.53–0.97	0.031	53%	0.007
Presence of PDA was assessed in selected infants	7	0.68	0.54–0.87	0.002	55%	0.036

k: number of studies; OR: odds ratio; CI: confidence interval; SGA: small for gestational age; IUGR: intrauterine growth restriction; GA: gestational age; MD: mean difference; BW: birth weight; PDA: patent ductus arteriosus.

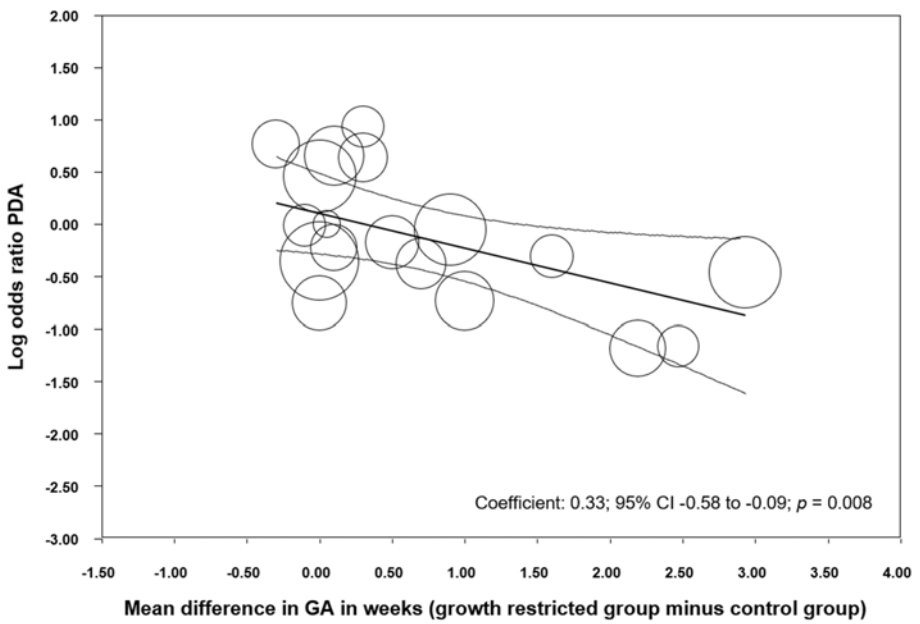

FIGURE 4 | Meta-regression of mean difference in gestational age (GA) between growth restricted and control groups and risk of developing patent ductus arteriosus (PDA). CI: confidence interval.

between SGA/IUGR and RDS ($p = 0.287$, R^2 analog: 0.13, **Supplementary Figure 7**).

DISCUSSION

Meta-analysis of observational studies presents challenging methodological issues involving differences in the design of the studies (i.e., cohort and case-control), assessment of exposure and outcomes, and control for potential confounders (24, 71–73). One of the main difficulties in understanding the relationship between IUGR and PDA is the lack of agreement among clinicians and investigators as to what defines these two clinical entities. In the present meta-analysis, we observed a significantly reduced rate of PDA in the SGA/IUGR group when all the studies were pooled. However, analysis of the subgroups with a more refined definition of either growth restriction or PDA could not confirm the association between the two conditions. In addition, we detected that some studies may be biased by including infants with lower GA in the control group.

Very recently, using the Delphi procedure, a consensus definition of neonatal growth restriction was reached by an international panel of pediatric leaders in the field (10). It was proposed to use the term "growth restriction in the newborn" to differentiate neonatal growth restriction from fetal growth restriction and SGA because, despite the overlapping among these terms, infants defined by them are not the same (10). The consensus proposed that the "use of a unique term will promote clarity in the categorization of infants, both in clinical practice and research, and will prevent conflation and confusion with SGA" (10). Growth restriction in the newborn was defined by a BW below the 3rd percentile on population-based or customized growth charts or at least 3 out of 5 of the following: BW <10th

percentile; head circumference <10th percentile; length <10th percentile; prenatal diagnosis of FGR; and maternal pregnancy information (e.g., hypertension or preeclampsia) (10). Therefore, a BW below the 10th percentile is not considered sufficient to define neonatal growth restriction (10). Interestingly, meta-analyses of the studies in which SGA was based on BW below the 10th percentile or percentile was not clearly specified were the only analyses showing a significant reduced risk of PDA in the SGA infants. In contrast, we did not observe a significant association between neonatal growth restriction and PDA when the studies using the 3rd percentile criteria and/or prenatal diagnosis of FGR were pooled. Therefore, when the growth restriction definition was refined, the negative association between growth restriction and PDA was not further observed.

Most studies included in the present meta-analysis used population-based BW references to define SGA infants. These references have been developed with large databases and provide BW percentiles by each GA (74–76). However, BW may not represent intrauterine growth trajectory at a given GA because preterm infants are more likely to be growth-restricted. Thus, the 10th or the 3rd percentile of the BW reference in very preterm infants are substantially lower than the corresponding percentiles of the ultrasound-based fetal weight reference (75). Consequently, it has been suggested that, when compared to ultrasound-based fetal weight references, population BW references significantly under-diagnose growth restricted infants in very preterm births (74–76). Alternatively, prescriptive and customized BW references have been proposed to improve the detection of growth restricted infants at higher risk of neonatal mortality and morbidity (10, 76). Prescriptive BW references are derived from infants who were not exposed to antenatal risk factors of FGR (76), whereas customized growth charts are population-based growth charts that have been adjusted for factors predicting BW such as maternal height and weight or ethnic group (10).

Besides the definition of the exposure (i.e., growth restriction), the definition of the outcome (i.e., PDA) is also controversial. Clear evidence is lacking for or against many of the current approaches to a PDA in very preterm infants. These uncertainties have resulted in different definitions of what is considered a "significant PDA" as well as in treatment strategies, which range from aggressive management to a more conservative approach, with some suggesting that the PDA is an innocent bystander to adverse outcomes (2, 3, 77–80). In 27 of the 47 included studies, PDA was defined as hemodynamically significant and/or PDA requiring treatment. When these 27 studies with a more refined PDA definition were pooled, meta-analysis could not show an association between significant PDA and neonatal growth restriction. In addition, we detected considerable heterogeneity concerning the time of and the indication for PDA assessment. Studies including only selected patients at high risk for PDA or with clinical findings suggestive of the condition may have different rates of PDA than studies screening all very or extremely preterm infants. However, subgroup analysis and meta-regression could not demonstrate that the OR for the association between SGA/IUGR and PDA is different between the studies with or without universal PDA screening. Unfortunately, the marked heterogeneity of the time of assessment of PDA did not allow us to perform any meaningful subgroup analysis on timing.

Failure to account for significant differences in baseline characteristics between groups in observational studies can lead to biased estimates. The major risk factor for PDA is low GA (1–4). We detected that in a substantial number of the included studies the SGA/IUGR group had a higher GA than the control group (see **Figure 3**). The difference in GA was particularly marked in the studies using BW, but not GA, as inclusion criterion. These facts may contribute to explain the "protective" effect of growth restriction on PDA development. Meta-regression analysis confirmed the correlation between the difference in GA between the SGA/IUGR and the control group and PDA risk. Moreover, subgroup analyses limited to studies without substantial difference in GA between the SGA and the control group could not find a significant association between SGA/IUGR and PDA. Zeitlin et al. assessed the prevalence of SGA age among 7,766 very preterm infants (GA below 32 weeks) from 11 European countries and observed that this prevalence was lower when GA was under 28 weeks (81). They speculate that this difference may reflect fewer indicated deliveries for growth restriction among extremely preterm infants (81). Etiology of very preterm birth (i.e., GA <32 weeks) can be divided into two main categories: infection/inflammation and dysfunctional placentation (82). However, the distribution of these two etiologies is not homogeneous. Infants with dysfunctional placentation are frequently less preterm and this etiology is strongly associated with growth restriction (82). In addition, very early mortality, which is a competing outcome for PDA, is also more frequent in the more preterm infants. Altogether this may explain the higher presence of older infants in the SGA/IUGR group. Nevertheless, when we performed a subgroup analysis that only included extremely preterm infants (GA <28 weeks), the results did not substantially differ from the meta-analysis including all preterm infants (**Table 2**, **Supplementary Figure 5**).

A common classical assumption among neonatologists is that the intrauterine stress associated with IUGR would accelerate lung maturation leading to a reduced rate of RDS (6). However, the evidence supporting this idea is scarce and, as reviewed by Rosenberg (6), even a number of studies reported a significantly increased risk of RDS in very preterm infants with IUGR. The relationship between PDA and RDS in very preterm infants is complex and bidirectional. In many instances, the presence of a hemodynamically significant PDA is suspected only on the basis of respiratory findings, such as increasing requirements for supplemental oxygen or mechanical ventilation (83). Conversely, changes in pulmonary precapillary tone as consequence of RDS evolution and/or therapy can alter the left-to-right PDA shunt (83). Therefore, we aimed to analyze the possible role of RDS on the association between PDA and neonatal growth restriction. Twenty-three studies included in our review reported on RDS rate and, when pooled, we observed that the protective effect against RDS was only present in the subgroup of studies using the definition of SGA based on BW below the 10th percentile. In addition, meta-regression could not demonstrate a significant correlation between the effect size of the association SGA/PDA and the effect size of the association RDS/PDA.

Besides the issue of the heterogeneity of definitions discussed above, our study has other limitations which deserve consideration. Only 3 studies examined growth restriction and PDA as their primary objective (13, 32, 69). Moreover, many cohort studies did not describe the role of GA or other confounders in infants with and without IUGR/SGA, which makes distinguishing the effect of IUGR from that of confounding factors difficult. Finally, for some definitions of SGA/IUGR only a limited number of studies could be included in subgroup analysis. The strengths of our study also deserve mention, including a comprehensive search, a large number of included studies, inclusion and data extraction by several researchers to reduce bias, and analysis of confounders through subgroup analyses and meta-regression.

CONCLUSION

The present systematic review and meta-analysis could not provide conclusive evidence on the association between neonatal growth restriction and PDA risk because of marked heterogeneity in definitions of both the insult and the outcome (i.e., significant PDA) as well as group differences in relevant baseline characteristics, such as GA, across the studies. An improved understanding of factors influencing the natural history of PDA and the risk factors for the condition may promote enhanced precision regarding diagnosis, monitoring, and treatment selection. Further investigation is needed to analyze whether specific conditions leading to neonatal growth retardation such as preeclampsia or pregnancy hypertensive disorders are associated with an altered risk of developing PDA or other complications in very preterm infants.

DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study can be found in the Hardvard Dataverse repository at: https://dataverse.harvard.edu/dataset.xhtml?persistentId=doi:10.7910/DVN/C0HYTD.

AUTHOR CONTRIBUTIONS

EV-M collected data and checked data for accuracy, planned and performed the statistical analyses, contributed to the interpretation of the results, drafted the final version of the manuscript, and reviewed and revised the manuscript. MK performed the search, selected studies for inclusion, collected data, contributed to statistical analysis, and drafted an initial version of the manuscript. PD selected studies for inclusion, collected data and supervised data collection, and reviewed and revised the manuscript. RC contributed to interpretation of results and reviewed and revised the manuscript. EV conceptualized and designed the study, contributed to the search, selected the studies for inclusion, supervised data collection, contributed to the statistical analyses and interpretation of the results, and reviewed and revised the manuscript. All authors approved the final manuscript as submitted.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00058/full#supplementary-material

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Conflict of Interest Statement: EV and RC were authors of studies included in the meta-analysis.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 08 February 2019
doi: 10.3389/fendo.2019.00046

Nutritional Intervention for Developmental Brain Damage: Effects of Lactoferrin Supplementation in Hypocaloric Induced Intrauterine Growth Restriction Rat Pups

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 14 November 2018
Accepted: 21 January 2019
Published: 08 February 2019

Citation:
van de Looij Y, Larpin C, Cabungcal J-H, Sanches EF, Toulotte A, Do KQ and Sizonenko SV (2019) Nutritional Intervention for Developmental Brain Damage: Effects of Lactoferrin Supplementation in Hypocaloric Induced Intrauterine Growth Restriction Rat Pups. Front. Endocrinol. 10:46. doi: 10.3389/fendo.2019.00046

Introduction: Intrauterine Growth Restriction (IUGR) refers to an impaired development of the fetus and hence results in adverse neurodevelopmental and psychiatric consequences later in life. Lactoferrin (Lf) is a glycoprotein present in milk that has already shown neuroprotective effects through its anti-inflammatory and antioxidant properties on impaired developing brains. The aim of this study was to characterize a rat model of IUGR and assess the neuroprotective effect of a nutritional supplementation with bovine Lf during pregnancy and lactation on this model.

Methods: A model of 50% gestational caloric restriction (CR) was used. Three groups were designed, and pregnant rats had either *ad libitum* access to food (control group, CTL) or 50% of the controls' intake (restricted group, IUGR). The diet was isocaloric and supplemented with bovine Lf for the caloric restricted dams (restricted-Lf, IUGR_Lf). At postnatal day 7 and 21, advanced *ex-vivo* diffusion MRI techniques at 9.4T were used to investigate brain cortical and white matter microstructure. Further, genes and proteins involved in structure (synaptophysin, MBP), microglia (Iba-1), metabolism (MCT2, βCaMKII) and apoptosis (Bcl-2) were analyzed in the cortex and striatum. In the cortex, the number of parvalbumin immunoreactive interneurons and their perineuronal nets were quantified. Behavioral tests were performed at P31.

Results: Effects of the CR were significant in the cortex and striatum with reduction of synaptophysin (marker of synaptogenesis) at P7 and MBP (marker of myelin) at P21 in the cortex. Indeed, MCT2 (energy metabolism), Bcl-2 (anti-apoptotic protein) and βCaMKII (synapse activity) expressions were reduced in IUGR groups at P7. In the striatum NG2 (marker of oligodendrocyte precursor cells) and Bcl-2 at P7 as well as βCaMKII at P21 were decreased following IUGR and restored by Lf. Cortical microstructure was impaired

following CR with partial effect of Lf. Lf prevented oxidative stress induced parvalbumin interneurons impairments whereas striatum and external capsule showed alterations in microstructure depicted by diffusion MRI, which were also partially reversed by Lf.

Discussion and Conclusion: The model of 50% caloric restriction induced mild impairment partially reversed by nutritional intervention using Lf during pregnancy and lactation.

Keywords: intrauterine growth restriction, caloric restriction, lactoferrin, neuroprotection, magnetic resonance imaging

HIGHLIGHTS

- Moderate undernutrition induced IUGR demonstrated mild cerebral impairment.
- Cerebral impairment is characterized by white and gray matters damage depicted by MRI.
- Oral Lf supplementation showed partial neuroprotection following caloric restriction during gestation.
- It represents a promising approach to reduce developmental diseases in IUGR and preterm infants.

INTRODUCTION

The definition of intrauterine growth retardation (IUGR) given by world health organization in 1995 was "An infant suffering from IUGR is defined as being below the 10% percentile of the recommended gender-specific birthweight for gestational age reference curves." It is one of the main causes of developmental brain damage with possible long life neurodevelopmental disabilities. If IUGR is an important public health concern worldwide, the rate of IUGR in the developing countries is about six times higher than in developed countries with at least 30 million IUGR infants per year. Poor antenatal and postnatal nutrition further prevents infants from attaining their full developmental potential. IUGR adults show a higher risk of altered cognitive and executive function and emotion control. The often-associated morbidities of IUGR, prematurity and poor postnatal growth represent a major risk for poor development of affective cognition and psychiatric disorders (1–3).

Lactoferrin (Lf), an iron-binding glycoprotein is involved in the intestinal iron uptake and regulates immune response with anti-inflammatory as well as antioxidant property (4, 5). In humans, higher concentration of Lf is found in the colostrum but Lf level decreases through lactation period (6). After oral administration, Lf is regulated through binding sites on brain endothelial cells allowing the passage from blood to tissues including the brain (7). Lf has been shown to down-regulate inflammation in lipopolysaccharides injury models (8). Importantly, Lf nutritional supplement in preterm shows significant reduction in late-onset sepsis and necrotizing enterocolitis (9). Our previous studies explored the effect of Lf on various animal models of perinatal brain injuries. Hypoxic-Ischemic post-natal day 3 (P3) pup rats demonstrated recovery with maternal Lf supplementation during lactation (10), characterized by a reduced cortical loss and an altered white matter recovery. Furthermore, inflammatory and apoptotic gene expressions were also reduced (10). Following lipopolysaccharides injection at P3, Lf administration during lactation reduced ventriculomegaly as well as impaired microstructure and white matter alterations (11). Finally, in a model of dexamethasone induced IUGR mimicking maternal stress and fetal growth restriction, the abnormal levels of brain metabolites as well as major neurotrophic factor (BDNF) were restored with Lf (12). Taken together, these findings suggest that Lf, with its anti-inflammatory and antioxidant properties, has great potential to reduce brain injury, improve cerebral development and prevent noxious programming due to adverse pre and postnatal conditions.

As such, the aim of this study was to evaluate structural and functional cerebral impairments in gestational caloric restriction induced IUGR as well as the potential neuroprotective effect of Lf supplementation. Effects of caloric restriction were assessed with advanced diffusion magnetic resonance imaging techniques [diffusion tensor imaging (DTI) as well as neurite orientation dispersion and density imaging (NODDI)] in conjunction with protein and gene expressions and behavioral tests.

MATERIALS AND METHODS

Animal Preparation

All experimental protocols have been previously approved by the Animal Research Ethics of Geneva, Switzerland (#GE7115). Nulliparous Wistar female (225–250 g) and Wistar male (250–275 g) rats (Charles River Laboratories, France) were used for breeding. During gestation, they had either *ad libitum* access to food: Control group (CTL) or access to 50% of the controls' intake: Restricted group (IUGR). Bovine lactoferrin (Lf) (Taradon laboratory, Tubize, Belgium) at an expected dose of 1g/kg/day according to daily intake of food by gestating and lactating dams, was added to the diet as a supplementation also during gestation and lactation for the Restricted-Lactoferrin group (IUGR_Lf). From the day before the parturition and during the whole lactation, all dams had *ad libitum* access to food, with either Lf enriched (1 g/kg/day) or isocaloric diet. Standard animal housing conditions according to the animal facility of the CMU, University of Geneva, were applied with free access to water. At birth, rat pups were culled or boarded out to another dam of the same group in order to control litter size to 10 to 12 pups per dam until weaning day. Dam and pup weight gain were measured daily until weaning and once a week until P42.

The number of pups per group is mentioned by experiment separately. The number of dams used in the study was: 6 controls, 10 IUGR and 9 IUGR_Lf.

Brain Collection for *ex-vivo* MRI and Immunofluorescence

After intraperitoneal injection of pentobarbital (50 μg/kg), rat pups were intra-cardially perfused with 0.9% NaCl and 4% paraformaldehyde solution at postnatal day 7 (P7) and 21 (P21). Brains were removed and immersed in 4% paraformaldehyde overnight for post fixation.

Magnetic Resonance (MR) Experiments

All MR experiments were performed on an actively-shielded 9.4T/31 cm magnet (Varian/Magnex) equipped with 12-cm gradient coils (400 mT/m, 120 μs). *Ex-vivo* MRI was performed on P7 (CTL; $n = 6$, IUGR; $n = 6$, and IUGR_Lf; $n = 6$) and P21 (CTL; $n = 7$, IUGR; $n = 4$, and IUGR_Lf; $n = 4$) fixed brains with a 2.5 mm diameter birdcage coil. A multi-b-value shell protocol was acquired using a spin-echo sequence with a field-of-view equal to 21 × 16 mm^2 and a matrix size of 128 × 92. Twelve slices of 0.6 mm thickness were acquired with 3 averages and TE/TR = 45/2,000 ms. Ninety-six diffusion weighted images were acquired including 15 b_0 images and 81 images separated in 3 non-collinear shells with a uniform distribution in each shell. Distribution of number of directions and *b*-value were as follow: 21 directions at a *b*-value of 1,750 s/mm^2, 30 directions at a *b*-value of 3,400 s/mm^2 and 30 directions at a *b*-value of 5,100 s/mm^2. NODDI toolbox (13) was used to fit the acquired data. Regions of interest were delineated in cortex (Cx), external capsule (EC), and striatum (St). DTI derived parameters [Axial diffusivity (AD), Radial diffusivity (RD), Mean diffusivity (MD) and Fractional anisotropy (FA)] as well as NODDI derived parameters [intra-neurite volume faction (f_{icvf}), isotropic volume fraction (f_{iso}) and orientation dispersion index (ODI)] were averaged in the different regions assessed as previously used in animal models of perinatal brain injuries (11, 14).

Tissue Collection for Protein and Gene Expression

Rat pups were euthanized by decapitation at P7 and P21. Pups were anesthetized with pentobarbital (50 μg/kg) prior to decapitation. Brains were quickly removed, dissected on ice and frozen instantly in liquid nitrogen. Two structures were dissected: cortex (Cx) and striatum (S). The samples were stored at −80°C until analyses.

Immunoblotting

Brain tissues from P7 (CTL; $n = 6$, IUGR; $n = 6$ and IUGR_Lf; $n = 7$) and P21 (CTL; $n = 10$, IUGR; $n = 14$ and IUGR_Lf; $n = 13$) rats were homogenized by sonication in RIPA buffer (Cell Signaling, 9806S), and the protein concentration was assessed using a Bradford assay. Proteins (25 μg) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by immunoblotting. All antibodies (**Table 1**) were diluted in blocking buffer containing 0.1% casein (Sigma-Aldrich, C8654). Antibodies were diluted in the concentrations suggested on the data sheet by the manufacturer (**Table 1**). After incubation of the primary antibodies, the following secondary antibodies were applied: goat anti-mouse IgG (H+L) conjugated with Dylight™ 680 (#5470, Cell Signaling Technology), goat anti-rabbit IgG conjugated with IRDye 800 (926-32210, LI-COR) and donkey anti-goat IgG conjugated with IRDye 680 (926-68074, LI-COR). The Odyssey Infra-red Imaging system (LI-COR) was used to visualize the protein bands. The densitometry was assessed by normalizing the optical density of each sample with respect to actin expression (mouse monoclonal anti-actin from Millipore, MAB1501), using Image Studio Lite ver.5.2 software. The results are expressed as a percentage of values obtained either for the control or for the restricted rat pups (100%) using actin as a standard.

Real-Time Quantitative PCR (RT-qPCR)

The extraction of the total mRNA was done using the RNase Mini Kit (Qiagen, 74104) following the manufacturer's instructions (**Table 2**). On P7 collected brains, 3 μg mRNA from the three regions of interest at P7 (CTL; $n = 6$, IUGR; $n = 6$ and IUGR_Lf; $n = 6$) and P21 (CTL; $n = 6$, IUGR; $n = 8$ and IUGR_Lf; $n = 8$) were reverse transcripted to cDNA using 400 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen, 28025-013), 20 units of recombinant RNAsin (Promega, N2515), 0.5 μg of random hexamers (ThermoFischer Scientific, #S0142), 2 mmol/L dNTP (Invitrogen, 10297018), and 40 mmol/L of dithiothreitol (Invitrogen, 18080093). Real-time quantitative PCR was performed with the PowerUp SYBR Green Master Mix (Applied Biosystems, A25742) and using an StepOnePlus™Real-Time PCR System (Applied Biosystems). Gene expressions were normalized using the housekeeping ribosomal gene RPS29. The results were calculated using the Livak approach and are expressed in arbitrary units (A.U) (12).

Quantification of Mitochondrial Oxidative Damage and Parvalbumin Immunoreactive Interneurons

Coronal frozen sections (40 μm) were used to investigate the anterior cingulate cortex (ACC), a region in the prefrontal cortex known to be sensitive to stress. Triple immunolabeling for labeling DNA oxidative damage, parvalbumin (PV), and perineuronal nets (PNNs) was performed as followed. An antibody against 8-oxo-2′-deoxyguanosine (8-oxo-dG), a product of DNA oxidation, was used as DNA oxidative marker, while the PNNs were labeled with the lectin *Wisteria floribunda Agglutinin* (WFA), which binds the N-acetylgalactosamines in chondroitin sulfate proteoglycans (15, 16). Brain sections containing the anterior ACC were first incubated with PBS + Triton 0.3% +sodium azide (1 g/L) containing 2–3% normal horse serum, then placed for 48 h in a solution with a mouse monoclonal anti-8-oxodG (1:400; AMS Biotechnology, Switzerland) and a rabbit polyclonal anti-parvalbumin (PV) (1:2,500; Swant Inc., Switzerland) primary antibody together with the biotin-conjugated WFA (1:2,000; Sigma, Switzerland). Sections were then washed, incubated with fluorescent secondary antibody conjugates: goat anti-mouse Alexa 488 (1:300; Life

TABLE 1 | Primary antibodies for immunoblotting including catalog numbers, companies, and dilutions.

Abbreviations	Primary antibodies	Catalog number/Company	Dilution
Actin	Anti-actin antibody clone C4	MAB1501/Millipore	1/1,000
CD68	Rabbit polyclonal anti-CD68	250594/Abbiotec^{TM}	1/500
DCX	Rabbit polyclonal anti-DCX	ab18723/Abcam	1/1,000
GFAP	Mouse monoclonal anti-GFAP	G6171/Sigma-Aldrich	1/1,000
Iba-1	Goat polyclonal anti-Iba1	ab5076/Abcam	1/1,000
MBP	Mouse monoclonal anti-MBP	ab62631/Abcam	1/1,000
NeuN	Mouse monoclonal anti-NeuN	MAB377/Millipore	1/1,000
NG2	Rabbit polyclonal anti-NG2	ab129051/Abcam	1/1,000
Synaptophysin	Mouse monoclonal anti-synaptophysin	ab8049/Abcam	1/500
βCaMKII	Rabbit polyclonal anti-CAMKII beta	ab34703/Abcam	1/1,000
DMT1	Rabbit polyclonal anti-DMT1	ab140977/Abcam	1/1,000
GLT1	Guinea pig polyclonal anti- GLT1	AB1783/Millipore	1/500
Leptin receptor	Rabbit polyclonal anti-Leptin Receptor	ab5593/Abcam	1/1,000
MCT2	Rabbit polyclonal anti-MCT2	AB3542/Millipore	1/1,000
NMDAR	Rabbit polyclonal anti-NMDA R2A	ab14596/Abcam	1/1,000
IGF 2	Rabbit polyclonal anti-IGF2	ab9574/Abcam	0.2 μg/mL
TrkB	Rabbit polyclonal anti-TrkB	ab18987/Abcam	1/1,000
Fractin	Rabbit polyclonal anti-Fractin	AB3150/Millipore	1/1,000

TABLE 2 | PCR primers for quantitative real-time PCR.

Primer	Forward	Reverse
Bax	5′-TGGAGCTGCAGAGGATGATTG-3′	5′-GCTGCCACACGGAAGAAGAC-3′
Bcl-2	5′-TGGGATGCCTTTGTGGAACT-3′	5′-GAGACAGCCAGGAGAAATCAAAC-3′
BDNF	5′-AAAAAGTTCCACCAGGTGAGAAGA-3′	5′-GCAACCGAAGTATGAAATAACCATAG-3′
RPS29	5′-GCCAGGGTTCTCGCTCTTG-3′	5′-GGCACATGTTCAGCCCGTAT-3′

Technologies, USA), goat anti-rabbit CY3 (1:300; Chemicon International, USA) and streptavidin 405 conjugate (1:300; Millipore Corporation, USA). Sections were visualized and processed with a Zeiss confocal microscope equipped with ×20 and ×40 Plan-NEOFLUAR objectives. All peripherals were controlled with LSM 710 Quasar software (Carl Zeiss AG, Switzerland). With the triple immunolabeling of 8-oxodG, PV, and WFA, Z-stacks of 12 images with ×20 objective (with a 1.8 μm interval) were scanned (1,024 × 1,024 pixels). Images were filtered with a Gaussian filter to remove background noise and sharpen cell profile contour. Only the inner 9 images of Z-stacks were used for the analyses. The stack images were merged in a tif file with Imaris. Analyses were performed in a delineated region of interest (ROI) comprising the ACC (mainly cg1 and small portion of cg2) by an observer unaware of the experimental groups. To quantify the overall 8-oxo-dG, the staining intensity and number of labeled voxels within the ROI were measured. For PV-IR cell count, PV cell bodies were counted and their mean intensity of labeling (arbitrary unit) also obtained. To analyze the number of PV+ cells surrounded by WFA labeled PNN (WFA+ PV), we used the same "spots module" used to count PV+ cells to assign spot markings on the profile-labeled voxels that fall within a given size. Briefly, the channels for PV and WFA immunolabeling were chosen, and the profile size criterion (>8 and >5 mm, respectively) was defined to quantify stained profiles above these given sizes. The procedure was visually monitored/verified before proceeding. Spots generated for PV that contacted/overlapped with spots generated for WFA (PNN) were considered as those PVI surrounded by PNN (WFA-positive PVI).

Behavioral Tests

Behavioral tests were performed from P31 to P41. Detailed protocol can be found in (17).$

The Elevated Plus Maze (EPM)

The test assesses anxiety-like behavior (17). Rats were placed in the central area, head facing one of the enclosed arms. Number of entries in the open or closed arms, time spent on each arm, head-dipping and rearing were recorded over 5 min to investigate anxiety-like state. The ratio based on the time spent in the closed arms and the time spent in the open spaces (open arms and center of the apparatus) was calculated by time in open space divided by time in closed arms (18).

The Open Field (OF)

Motor activity, exploratory drive, and anxiety state were assessed in this test in a circular arena (100 cm diameter) divided in 21

areas. The latency to leave the central circular area, the number of areas crossing and rearing were recorded for 5 min (17).

The Novel Object Recognition (NOR)

The novel object recognition (NOR) test was performed in the open field arena during two consecutive days. In the first day, each rat was confronted with two identical objects (A and A′) placed 20 cm apart from the walls of the apparatus. The time exploring each object was recorded for 4 min. In the second day, long-term memory was tested by exposing each animal to two objects in the same open field apparatus: one of the objects used in the first day, considered as the familiar object (A) and a novel (and distinct from A or A′) object (B). Object exploration time for each object was measured and a discriminative index (DI) was calculated by exploration time on object B minus exploration time on object A divided by total exploration time on both objects (19).

The Beam Walking (BW)

The beam walking (BW) test was composed of training phase and testing phase. In the first day, rats were trained (three trials) to traverse a wooden beam (width 2 cm, length 100 cm, elevated 70 cm above the floor). They were placed on one side and encouraged to walk to reach a black box on the other side of the beam. In the next day (24 h later) animals were exposed to the same beam and number of hind paw slips were counted as an index of locomotor deficits (17).

The Morris Water Maze (MWM)

The Morris water maze (MWM) was done for 6 days (5 days of training and 1 day of test—namely Probe Trial) consecutively in a black tank (200 diameters) filled with water (45 cm depth) at 21 ± 2°C. A black platform (diameter 10 cm) was placed at 2 cm under water level and remained hidden at the same location for the 5 training days. Rats underwent four trials with 10 min inter-trial interval per day and entered the pool facing the wall following a randomized sequence of the four starting points (North, East, South, and West). Latency to find the platform was measured up to 60 s and was considered as a learning indication. If the animal failed in finding the platform within the 60 s, it was conducted to the platform by the experimenter and left during 10–15 s for exploring the room clues. On the 6th day, the probe test trial day, the platform was removed and latency to cross the platform zone and time spent in the platform quadrant were assessed.

Statistics

For statistical analysis, a one-way ANOVA test followed by Tukey *post hoc* was done for normally distributed data. A Kruskal–Wallis test followed by a Mann–Whitney test ($P < \alpha$/number of tests) was done in case of non-normal distribution. Finally, the mean number of PV+ cells, WFA+PVI, and the overall 8-oxo-dG labeling intensity were compared between the three experimental groups and analyze with ANOVA test followed by a Dunnett test. Significance level was considered when $P < 0.05$ after rectification of *P*-values by Bonferroni correction. All data are presented as mean ± SEM except DTI and NODDI derived parameters as well as PV and WFA cells results expressed as mean ± SD.

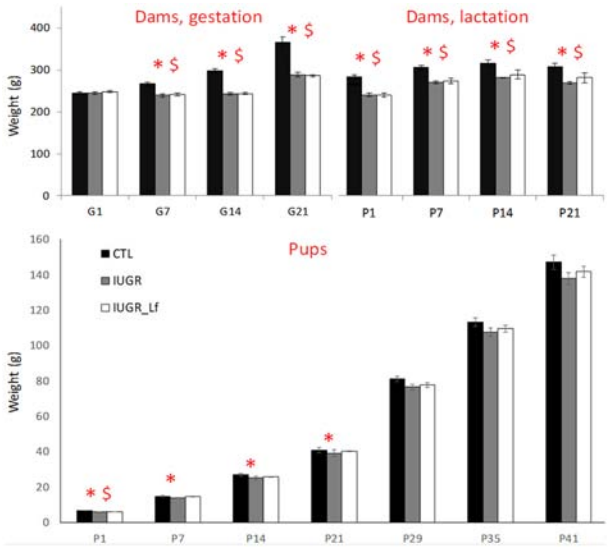

FIGURE 1 | High: dam weight monitoring through gestation (G1 to G21) and lactation until weaning (P1 to P21). Low: rat pups weight monitoring from post-natal day 1 to 41 (P1 to P41). Results are mean ± SEM. $P < 0.05$ *CTL vs. IUGR, $CTL vs. IUGR_Lf.

RESULTS

Caloric Restriction Effects on Maternal and Offspring Weights

In order to evaluate the impact of a 50% caloric restriction on dams, weights were measured daily during both gestation and lactation (**Figure 1**). Maternal body weights were significantly decreased in IUGR and IUGR_Lf groups compared to CTL group. The difference persisted postnatally until weaning. While IUGR and IUGR_Lf gained only 17% of initial weight at gestational day 21 (G21), whereas CTL dams gained 50% of initial weight. No significant effect of Lf supplementation was perceived on the IUGR_Lf dams compared to the IUGR ones. However, IUGR_Lf dam body weights were no longer statistically different from the control groups at the third week of lactation.

At P1 (**Figure 1**), IUGR and IUGR_Lf pups were lighter than CTL pups. At P7 and 14, only IUGR pup weights were different from CTL group. From P21, pups grew with no significant difference among them.

Microstructural Integrity

To allow the assessment of cerebral microstructure, diffusivity (Mean, MD; Axial, AD and Radial, AD), fractional anisotropy (FA) and direction encoded color maps (DEC), intra-neurite volume fraction (f_{icvf}), cerebrospinal volume fraction (f_{iso}) and orientation dispersion index (ODI) maps acquired at P7 and P21 on a 9.4T scanners are presented in **Figure 2**. High Signal-to-noise ratio and very good resolution were obtained leading

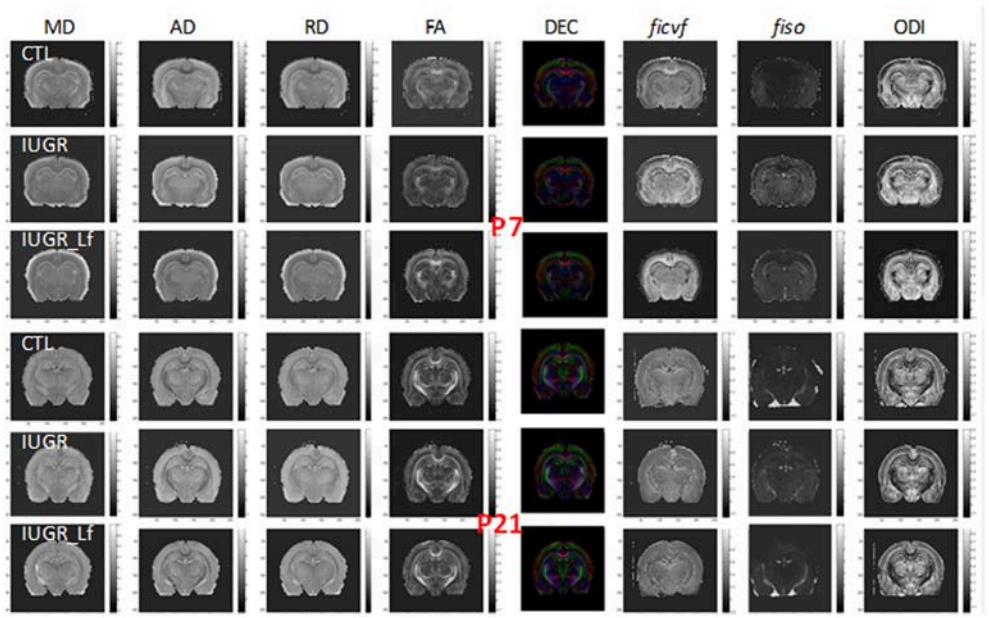

FIGURE 2 | Diffusivity (Mean, MD; Axial, AD; Radial, RD), fractional anisotropy (FA) and direction encoded color (DEC) maps, intra-neurite volume fraction (f_{icvf}), cerebrospinal volume fraction (f_{iso}), and orientation dispersion index (ODI) maps at P7 and P21 for each group.

to accurate measurement of the DTI and NODDI derived parameters (20).

Cortical Microstructure

Cortical DTI and NODDI derived parameters assessing cortical microstructure integrity, were obtained by diffusion MR imaging (**Figure 3**). IUGR and CTL derived parameters were not different from each other at P7, but IUGR_Lf parameters were different compared to the IUGR group: Indeed, a significant decrease in radial (RD) and mean diffusivity (MD), and also increased fractional anisotropy (FA) and intraneurite volume fraction (f_{icvf}) were observed with Lf. At P21, caloric restriction induced significant increase of RD, MD and f_{iso}, this last parameter was not restored by Lf.

In the cortex, most of the cell markers were not modified (**Figure 4**). At P7, only synaptophysin expression was significantly decreased in the IUGR group compared to CTL, showing a potential delay in synaptogenesis (**Figure 4**) but expression was similar between IUGR and IUGR_Lf cortices (**Figure 4**). At P21, only MBP expression was different among the groups (**Figure 4**). This marker of myelin formation and compaction was significantly decreased in the IUGR group. In the IUGR_Lf group, MBP expression was not statistically different from both IUGR and CTL groups (**Figure 4**). Also, Iba-1 expression was increased in IUGR pups, suggesting an impact on microglia. However, no alteration in CD68 expression, as marker of reactive microglia, expressions in the IUGR_Lf group were not different from the IUGR group at both ages (**Figure 4**).

Key receptors, transporters and kinases taking part in the global cerebral metabolism were quantified (**Figure 5**). At P7, MCT2 cortical expression was significantly decreased in the IUGR group (**Figure 5**), probably as a consequence of disrupted energy metabolism. Also, at P7, a trend was visible with an increased expression of leptin receptor in IUGR pups when compared to CTL pups ($P = 0.055$). βCaMKII, a major kinase in pre- and postsynaptic mechanisms and related to synaptic plasticity, was statistically less expressed in P7 IUGR pups, and showed a trend to be more expressed in IUGR_Lf animals (**Figure 5**, $P = 0.086$). No statistical difference was observed in the IUGR_Lf group (**Figure 5**). Protein and gene expressions involved in growth, differentiation and survival, as well as in apoptotic pathways were analyzed by immunoblotting and RT-qPCR. Cortical mRNA levels of BDNF showed a trend ($P = 0.068$) to be decreased (**Figure 5**) and the Bcl-2 mRNA level was significantly decreased in the P7 IUGR group (**Figure 5**), whereas no statistical difference with IUGR_Lf was observed (**Figure 5**) compared to CTL. Fractin expression, known to attest caspase 3 activity, was not statistically different among the groups (**Figure 5**).

At P21, we assessed whether a hypocaloric induced IUGR would induce oxidative stress in the brain of rat pups, leading to impairment of the cortical parvalbumin (PV) expressing neurons, known to play a critical role in cognition. A significantly higher 8-oxo-dG [marker for DNA oxidative damage (21)] in the cortex of hypocaloric induced IUGR pup rats compared to control was observed ($P < 0.0001$, **Figure 6**). We have previously established that oxidative stress during development leads to PV-IR and WFA+ PV cell deficit (22, 23). Indeed the number of PV-IR cell and PV-IR cell intensity in the ACC of IUGR rats were significantly decreased compared to CTL (**Figure 6**, # of PV cell: $P = 0.0044$, and PV-IR cell intensity: $P = 0.047$, not shown).

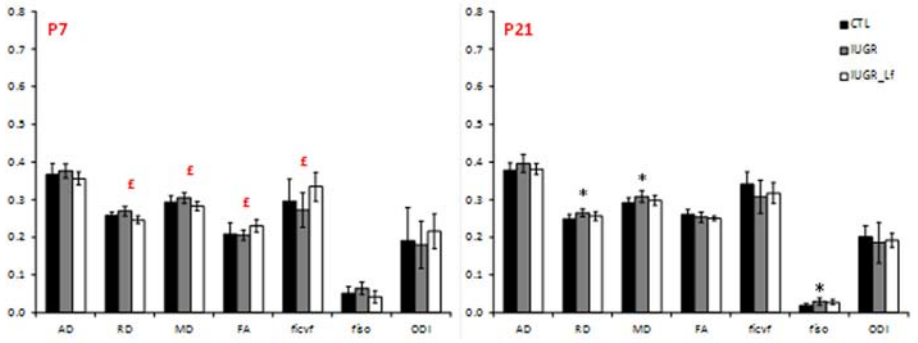

FIGURE 3 | Histogram of diffusivities (Mean, MD; Axial, AD and Radial, RD; $\times 10^{-4}$ $mm^2.s^{-1}$), fractional anisotropy (FA), intra-neurite volume fraction (f_{icvf}), cerebrospinal volume fraction (f_{iso}), and orientation dispersion index (ODI) at P7 and P21 in the cortex. Results are mean ± SD. $P < 0.05$, *CTL vs. IUGR, $CTL vs. IUGR_Lf, £IUGR vs. IUGR_Lf. *Effect of the lesion; £effect of Lf, red positive, black negative; *, £effect of the lesion restored by Lf; *,$effect of lesion but no effect of Lf; *, £,$effect of the lesion partially restored by Lf.

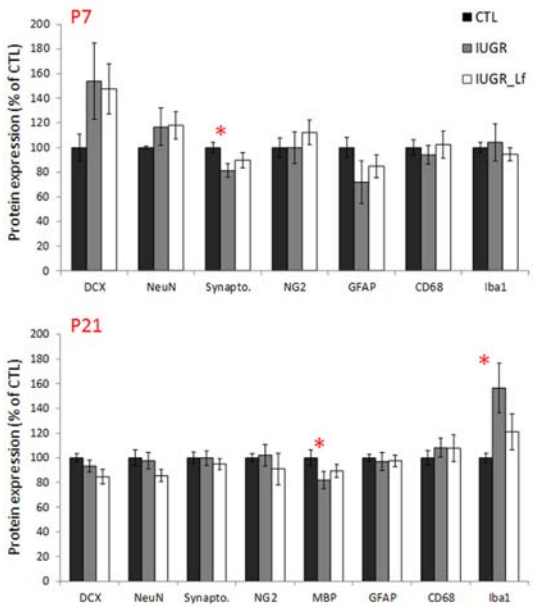

FIGURE 4 | Normalized protein expression to the control group level in CTL, IUGR, and IUGR_Lf pups at P7 (high panel) and P21 (low panel) in the cortex. Results are mean value ± SEM. $N = 5$–7 pups per group (P7) and $N = 9$–14 pups per group (P21). $P < 0.05$ *CTL vs. IUGR. Raw data in **Supplementary Tables 1, 2**.

However, the WFA+ PV cells, which are PV+ cells surrounded by PNN were not strongly affected as the decrease in IUGR rats *vs.* CTL remained at a trend level.

In order to assess the subsequent damages of oxidative stress on PV-IR cells and WFA+ PV (PNNs) during early post-weaning ages, we assessed whether Lf could prevent PV-IR and PNN impairment in ACC of IUGR rats. Indeed, Lf supplementation during development prevented increase of 8-oxo-dG intensity in the ACC of IUGR_Lf rats as compared to IUGR rats ($P < 0.0001$, **Figure 7**). The oxidative stress marker levels, 8-oxo-dG intensity, in ACC of IUGR_Lf rats did not significantly differ from that of in control rats. Lactoferrin also fully restored the number of PV-IR cells in ACC of IUGR_Lf compared to IUGR alone ($P = 0.001$), and compared to CTL rats ($P = 0.65$, NS). Interestingly, the WFA+ PV (PV PNNs) deficit in ACC of IUGR rats was not prevented by Lf ($P = 0.23$, NS).

Striatum

DTI and NODDI derived parameters were measured in the striatum (**Figure 7**). IUGR striatal microstructure integrity presented significant alterations compared to the CTL group. Increase in AD and RD at P7 and P21, as well as decrease in f_{icvf} and increase in f_{iso} at P7 depicted microstructural changes. DTI and NODDI parameter changes were reverted in P7 IUGR_Lf pups and partially at P21.

Structural proteins used as cell markers in the striatum were not differentially expressed between the groups. However, NG2 expression was increased at P7 in IUGR_Lf striatum compared to the IUGR group (**Figure 8**). In striatum, at P7, the apoptotic pathway was potentially affected in IUGR pups with the anti-apoptotic Bcl-2 mRNA expression statistically downregulated and data showed a tendency ($P = 0.053$) to be reversed in IUGR_Lf when compared to IUGR pups. At P21 (**Figure 8**, lower panel), alteration in synaptic plasticity in the IUGR group was depicted by a statistical decrease in βCaMKII expression compared to the CTL group. This altered expression was reverted in the IUGR_Lf group. Fractin quantification was not statistically different among the groups. Growth factor IGF2 was found to be statistically increased in IUGR at P21 with no statistical difference in IUGR_Lf.

External Capsule

DTI and NODDI derived parameters in external capsule white matter tract are shown in **Figure 9**. At P7, RD was significantly increased in IUGR pups and reversed, along with a decrease in AD and MD, in IUGR_Lf when compared to IUGR. In the P7 IUGR_Lf group, f_{icvf} and f_{iso} were also significantly different from the IUGR group and there was no statistical difference between IUGR_Lf and CTL pups.

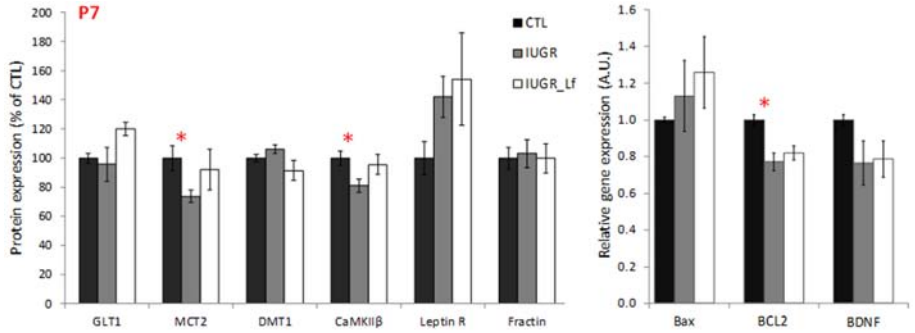

FIGURE 5 | Cortical protein (normalized to the control group level) involved in global brain mechanisms such as metabolism, growth and apoptosis for CTL, IUGR, and IUGR_Lf pups at P7. Quantification was done either after immunoblotting or RT-qPCR. Selection of metabolic transporter and receptor expressions (GLT1, MCT2, DMT1, CaMKIIβ, and Leptin R) and proteic feature of apoptosis (Fractin). Expression of pro- and anti-apoptotic mRNA (Bax and BCL2) and growth factor BDNF mRNA expression (BDNF). Results are mean values ± SEM of $N = 6–7$ pups per group. $P < 0.05$ *CTL vs. IUGR. Raw data in **Supplementary Table 3**.

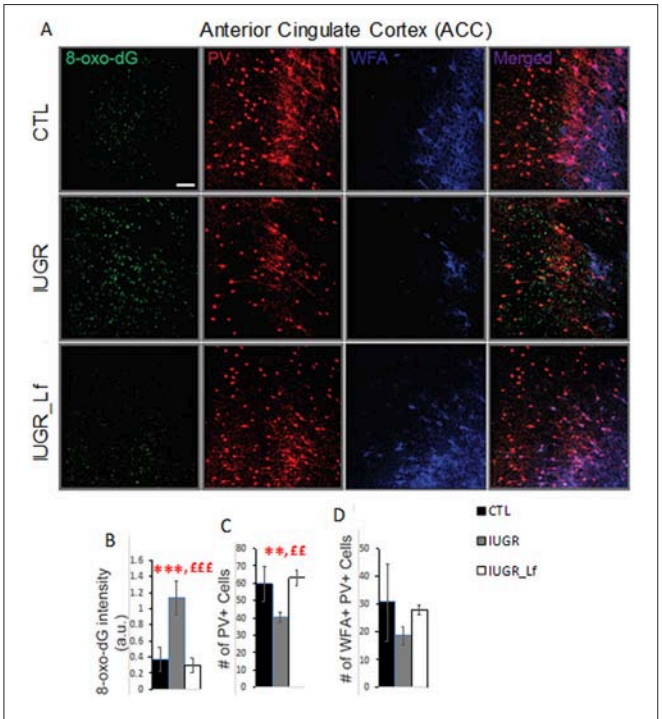

FIGURE 6 | Interneurons assessment in the cortex at P21. **(A)** Micrographs showing labeling for 8-oxo-dG (labels mitochondrial DNA oxidation), PV, and WFA (labels PNN) in ACC of CTL, IUGR, and IUGR_Lf. **(B)** Lactoferrin prevents increase in 8-oxo-dG labeling. **(C)** Quantification reveals that Lactoferrin restores PV immunoreactive cell deficits in hypocaloric induced IUGR rats. **(D)** There is a tendency for lactoferrin to prevent decrease of PNN (WFA + PV) in hypocaloric induced IUGR rats. Bars in all graphs represent SD. For each group $n = 4$ (for each animal 3–4 brain sections). *CTL vs. IUGR, £IUGR vs. IUGR_Lf, number of * or £: 3 $P < 0.001$; 2 $P < 0.01$; Scale bar: 50 μm.

Behavioral Tests

Cognitive and motor functions were assessed by a battery of behavioral tests performed between P31 to P41. These tests target behavioral aspects that could be impaired in IUGR children, such as motor activity, attention, memory and anxiety. Rats were frequently observed and monitored; pups from IUGR groups did not exhibit any abnormalities in motion, activity or basic behavior compared to the controls.

Elevated Plus Maze (EPM)

The elevated plus maze assesses the motor activity as well as the anxiety level. The global activity between the groups was not different and showed the three groups staying more in the closed arms than in the open ones (**Figure 10**). Ratio of time spent in the open to time spent in the closed arms did not show any difference among groups.

Open Field Test (OFT)

This test assesses the exploratory drive, as well as the global activity. Rats usually avoid open spaces and rather prefer exploring the area close to the wall (thigmotaxis). This trend was visible within the three groups (**Figure 10**). However, no differences were observed.

Beam Walking Test (BWT)

Locomotor activity and coordination were assessed by BWT. The number of paw slips was converted into digits and counted as number of mistakes. No statistical differences were found between the groups (**Figure 10**).

Novel Object Recognition (NOR)

The NOR test was used to assess long-term memory. Rats were confronted to two objects in the 2 sessions of testing: two familiar objects (*objects A and A′)* and a new one (*object B)*. The data did not detect preferences for the new objects nor differences within groups (**Figure 10**).

Morris Water Maze (MWM)

In the day of the test (Probe Trial) when the platform was removed, the time to find the platform zone was measured (**Figure 10**). CTL pups performed significantly better, crossing the platform region faster than IUGR and IUGR_Lf groups. The ability to learn did not vary between the groups during the training phase.

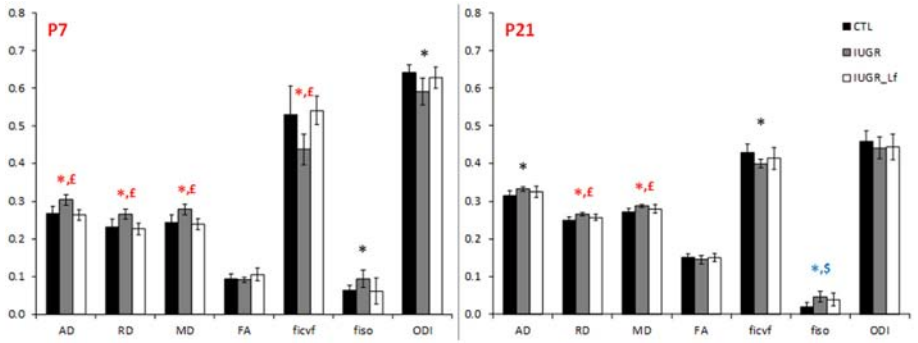

FIGURE 7 | Histogram of diffusivities (Mean, MD; Axial, AD and Radial, RD; $\times 10^{-4}$ $mm^2.s^{-1}$), fractional anisotropy (FA), intra-neurite volume fraction (f_{icvf}), cerebrospinal volume fraction (f_{iso}), and orientation dispersion index (ODI) at P7 and P21 in the striatum. $P < 0.05$, *CTL vs. IUGR, $CTL vs. IUGR_Lf, £IUGR vs. IUGR_Lf. *Effect of the lesion; £effect of Lf, red positive, black negative; *, £effect of the lesion restored by Lf; *,$effect of lesion but no effect of Lf; *, £,$effect of the lesion partially restored by Lf.

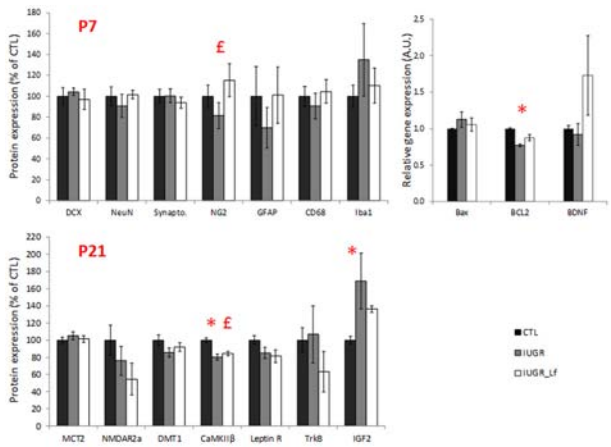

FIGURE 8 | Striatal protein (normalized to the control group level) for CTL, IUGR, and IUGR_Lf pups at P7 (high panel) and P21 (low panel). Structural protein expression at P7 (DCX, NeuN, Synapto., NG2, GFAP, CD68, and Iba1). Expression of striatal pro- and anti-apoptotic mRNA at P7 (Bax and BCL2). Selection of metabolic transporter and receptor expressions in the striatum at P21 (MCT2, NMDar2a, DMT1, CaMKIIβ, and Leptin R). Growth factor molecule and receptor mRNA expression in the striatum at P21 (TrKB and IGF2). Results are mean values ± SEM of $N = 3$ to 14 pups per group. $P < 0.05$ *CTL vs. IUGR, £IUGR vs. IUGR_Lf. Raw data in **Supplementary Tables 4, 5**.

DISCUSSION

In the present study, we explored the potential neuroprotective action of maternal bovine lactoferrin (Lf) supplementation on a 50% CR-induced IUGR model. As IUGR is thought to induce a delay in neurodevelopment, we addressed special focus on brain microstructural integrity, cell markers as well as metabolic proteins and gene expressions, which are important in cerebral development and injury. Further investigations were pursued for behavioral deficits. Analyses were performed at P7, P21 and from P31 to P41. P7 corresponds to the near term equivalent age for humans. P21 corresponds to the peak of myelination and cortical maturation providing already good information about the consequences of IUGR and potential Lf neuroprotection. Behavioral tests were performed at a time period where myelination is nearly completed (P31 to 41, preadolescent human equivalent) and as such potential mental disabilities can be observed in children at this time point. Indeed, these tests target behavioral aspects that could be impaired in IUGR children, such as motor activity, attention, memory and anxiety.

IUGR Model

Despite any effect of Lf supplementation on the weight of the dams, evidences validated our CR-induced IUGR model as it reduced maternal weight gain during gestation and had long-lasting effect during lactation period. While control dams gained 50% of their initial body weight by the end of gestation, restricted animals gained only 17%. This reduced maternal weight gain was also observed in a previous study using a stress induced model (maternal dexamethasone infusion) during the third week of gestation (12) and by others (24). Low birth weight in IUGR newborns is considered as a major indicator of prenatal insults. Pups were weighted at P1, and as both restricted diets were isocaloric, it was not expected that nutritional supplementation with Lf would improve low body weight in P1 IUGR pups indicating that offspring birthweight is highly dependent on the maternal food caloric intake during gestation. However, while IUGR pups receiving Lf through lactation caught up control body weight at P7, IUGR catch-up was only observed at P21. This gives rise to a potentially improved early metabolism in IUGR pups receiving Lf. In a placental insufficiency model, pups with moderate IUGR had a catch-up growth at P7, whereas catch-up growth in severe IUGR pups was not reported (25).

Cortical Impairment and Effects of Lactoferrin

We reported a decrease in cortical synaptophysin expression at P7 in whole cortical homogenate tissue, resulting from caloric restriction. As IUGR neurodevelopment is thought to be delayed due to reduced nutrients during a critical

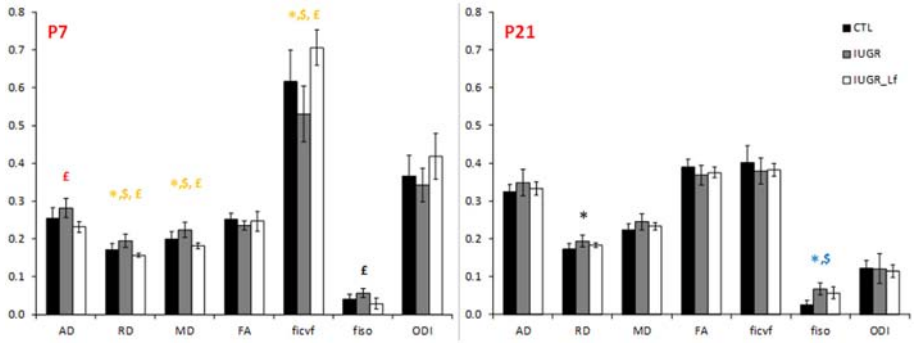

FIGURE 9 | Histogram of diffusivities (Mean, MD; Axial, AD and Radial, RD; $\times 10^{-4}$ $mm^2.s^{-1}$), fractional anisotropy (FA), intra-neurite volume fraction (f_{icvf}), cerebrospinal volume fraction (f_{iso}), and orientation dispersion index (ODI) at P7 and P21 in the external capsule. $P < 0.05$, *CTL vs. IUGR, $CTL vs. IUGR_Lf, £IUGR vs. IUGR_Lf. *Effect of the lesion; £effect of Lf, red positive, black negative; *, £effect of the lesion restored by Lf; *,$effect of lesion but no effect of Lf; *, £,$: effect of the lesion partially restored by Lf.

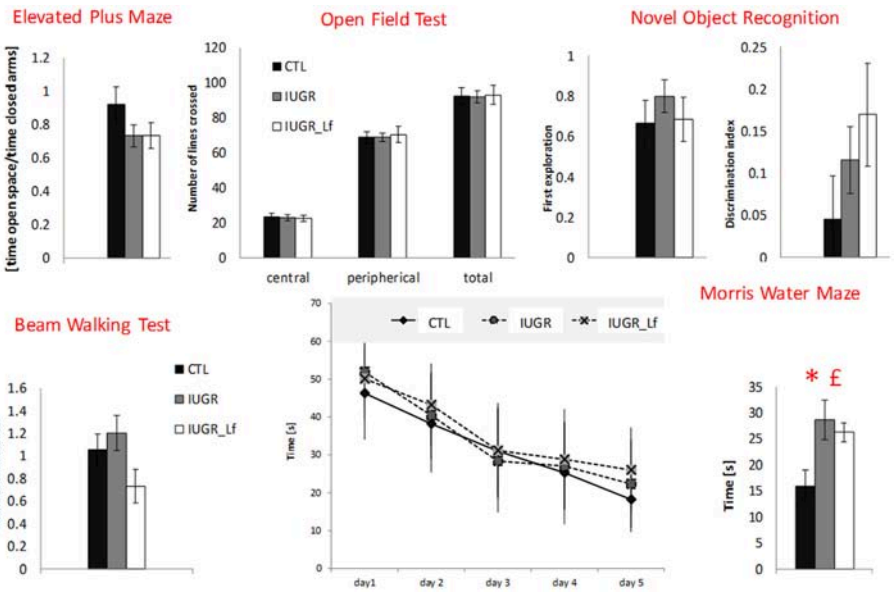

FIGURE 10 | Behavioral test results for CTL, IUGR, and IUGR_Lf pups. Elevated plus maze: ratio measuring the time spent in open space divided by the time spent in closed arm in the elevated plus maze. The center of the apparatus was considered as open space. Open field test: exploratory drive analysis by number of lines crossed in the central and peripherical zone, and in total in the open filed test. Novel object recognition: preferences between familiar and new object in novel object recognition. First object explored (familiar object = 0 and new object = 1) and Discrimination index showing preference for the new object. Beam walking test: number of paw slips during the beam walking test. Morris water maze: time to find the platform zone in the Morris water maze with learning curve displaying the time to find the platform through the five-day training (Day values are the mean of four trials) and time to find the platform zone the day of the test. All results are mean values ± SEM, $N = 16$–26 pups per group. $P < 0.05$ *CTL vs. IUGR, £IUGR vs. IUGR_Lf.

phase of cerebral development (26–28), the present result suggest that IUGR leads to transient delay in synaptogenesis in the cortex in P7 restricted rats. Similarly in sheep (29), impaired fetal environment was induced by an intra-amniotic lipopolysaccharide pro-inflammatory environment and cerebral synaptophysin expression was reported to be decreased in motor, somatosensory and entorhinal cortices causing reduction in density of presynaptical boutons.

A decrease in beta Ca2+/calmodulin-dependent protein kinase II (βCaMKII) expression in the cortex further supports this interpretation. βCaMKII is activated by calcium and is an important kinase targeting substrate responsible for long-term potentiation (30). *In vitro* overexpression induced an increase in the number of neurite extensions and in formation of new synapses (31). Therefore, it is related to morphology and quantity of synapses (32) by modifying the cytoskeletal structure. Along with our previous results on synaptophysin expression, decrease in βCaMKII emphasizes a potentially altered number/size of synapses and supports a transient delay in synaptic formation and possibly function.

Myelin basic protein (MBP) is one of the main components of myelin sheath structure and primordial for its formation and compaction and for axonal. In the current study, MBP expression was reduced in the cortex in IUGR pups at P21. Uterine artery ligation IUGR induced models have been widely used to approach white matter damage linked to perinatal impairments such as prematurity or fetal growth restriction observing a similar reduction in MBP density in the corpus callosum and the cingulum, sign of myelination defect (24).

Moreover, cortical expression of Iba-1, a microglial protein, was increased at P21. As it is expressed in both quiescent and amoeboid form and even though it is upregulated by activation (33), we quantified CD68 expression in order to identify properly activated microglial. However, CD68 analysis failed to identify microglial activation induced by IUGR. In a placental insufficiency rat model, inflammatory microgliosis was reported to impair white matter development (24). Indeed, using another marker (OX42) for immunohistochemistry, a higher density of microglia was observed in IUGR rats. Taken together, myelin integrity alteration concomitant with increased microglia density are in accordance with others work (14). Even though, our current results showed comparatively less microstructural alterations, it may further demonstrate the neuroinflammatory basis for cerebral impaired development in IUGR condition.

Despite an absence of significant differences, a tendency in the up-regulation of βCaMKII, synaptogenesis and MBP levels was observed in the Lf supplemented group providing evidence for a beneficial effect on cortical development. Indeed, DTI and NODDI derived parameters demonstrated a potential conservation of the microstructure in the cortex at P7 in IUGR_Lf rats compared to IUGR even though at P21 remaining impairments detected by diffusion MRI appeared moderates in the IUGR group. Altogether these results suggest that Lactoferrin reduces the delayed and impaired cortical development following caloric restriction.

We have demonstrated that redox dysregulation and oxidative stress (genetic or environmental origin) during development, in several animals models relevant to schizophrenia and autism, at microcircuit levels, leads to excitatory-inhibitory imbalance through impairment of the PV inhibitory network and their PNN (23, 34, 35). Similar redox dysregulation processes during development, at macrocircuit levels, could underlie impaired oligodendrocytes and delayed cortical myelination (34, 36). Parvalbumin immunoreactive (PV-IR) cell, oligodendrocyte and myelination impairment have been reported in schizophrenia (37). Abnormal control of redox system could therefore affect neuronal synchronization important for cognitive processing, and also disturb myelination leading to subsequent white matter injuries. N-acetyl-cysteine (NAC) is an antioxidant demonstrated to prevent oxidative stress during development and as such, to protect PVI and PNN. Clinically, several positive effects of NAC have been observed including an improvement of negative symptoms, a decrease of antipsychotics side effects, and an improvement of mismatch negativity and local neural synchronization in chronic schizophrenic psychosis. Recently, Conus et al. (38) observed on early psychosis patients that NAC add-on therapy for 6 months led to significant improvements in neuro-cognition and a reduced positive symptoms in patients presenting high oxidative status.

In the present study, lactoferrin prevents oxidative stress thus restoring PV-IR cell deficits in ACC of hypocaloric induced IUGR rat pups. Lactoferrin could have prevented oxidative stress and restored PV-IR impairment through anti-inflammatory and antioxidant properties in the same way as NAC does, even without improving MBP expression (myelination). Although beyond the scope of the present study, it may prove vital for the future studies to look into additional functional consequences of hypocaloric induced IUGR, such as mismatch negativity (sensory gating), and fast spiking neuronal synchronization (important for cognitive and social processing), and the effect of lactoferrin supplementation. This would certainly shed additional light on the contribution of a developmental undernutrition in psychiatric disease such as schizophrenia and potential prevention.

Impaired Microstructure in Striatum and Restoration With Lf

MR Imaging of the striatum of IUGR pups showed promising improvement of the neurodevelopment with Lf. The striatum is a vulnerable gray matter area in developing brain (11), which was proposed to be especially affected in case of periventricular leukomalacia and may be related to mild motor deficits (39) but also potentially to psychiatric disorders such as schizophrenia (40). In the present study, at P7, microstructural organization was altered or delayed, as depicted by reduction of the neurite density (f_{icvf}) and dispersion of the fibers (ODI) were reduced whereas isotropic volume fraction (f_{iso}) was increased. No changes in cell markers used in the current study were identified, suggesting alterations of arborization/maturation rather than in neuronal or glial reduced populations. No difference in MBP expression was observed, however, the low level of MBP immunostaining of brain sections at early time-point did not allow a structural assessment in the axonal fascicles. Nonetheless, these parameters were normalized with Lf, promoting a better integrity of the striatum in the first week after birth. Similar neuroprotective effect of Lf was observed at longer term (P21) in the striatum following lipopolysaccharide injection in the rat pup (11). MCT 2 expression in the striatum was decreased as observed in the cortex. Similar to the cortex, striatal mRNA expression of Bcl-2 was also downregulated and normalized with the Lf supplementation. As Bax expression was not affected by IUGR, reduction in anti-apoptotic protein presumed an increased apoptotic rate in IUGR condition (Bax/Bcl-2) (41). However, no increase in fractin expression was visible. Interestingly, βCaMKII protein expression was reduced in IUGR and restored with Lf at P21. Lactoferrin administration also improved striatal expression of βCaMKII. DTI/NODDI and βCaMKII findings suggest reduced arborization as consequence of IUGR reversed by Lf.

External Capsule, IUGR Impact on White Matter Tracts, and Repair With Lf

In external capsule, increased water diffusivity was observed perpendicularly to the main diffusion direction at P7. This

abnormal RD was associated with increased f_{iso} and reduced neurite density emphasizing clearly for a defective myelin organization. RD in the external capsule and corpus callosum were also increased in P21 moderate—but not in mild—IUGR pups after placental insufficiency (42). As it was correlated with poor oligodendrocyte density and an abnormal percentage of unmyelinated axons, RD modification was suggested to result readily from this myelin injury. All these studies related impaired neurodevelopment with alteration in the external capsule around 3 weeks of age. By this time, oligodendrocyte and astrocyte proliferations and maturations stage is likely to be finished. However, in our case, the microstructural alterations of external capsule were observed in P7 pups transiently as almost no difference was measured at P21. Positive effect of Lf at 7 days point was obvious. Our model further argues for a potential delay in myelination process rather than a long-lasting injury.

Neuroprotection with Lf was assessed in the inflammatory (11) and hypoxic-ischemic (10) models, as well as in the present CR model. While partial normalization was observed in the case of brain inflammation, diffusivities were fully normalized in the hypoxic-ischemic injured brain, such as the RD in the present study. Supplementation with Lf appeared to improve impaired white matter formation.

Limitation of IUGR Impact and Neuroprotection by Lf

The present study further illustrates the complexity of the IUGR condition, which depends on the timing, the severity, the heterogeneity of risk factors and pathways potentially impaired that are mimicked by various animal models. Maternal stress, placental insufficiency and undernutrition mimicking-models were all reported to interfere with fetal trajectory growth but altered pathways are very likely to differ depending on the risk factor (43). Our present model, moderate undernutrition, using a 50% caloric restriction during gestation, is likely to induce a mild and transient impairment to the neurodevelopment despite the IUGR phenotype. We believe that part of the non-significant changes seen in the model and the partial Lf neuroprotection are due to the mild IUGR model used. It is important to notice that mild IUGR has been also reported in humans with moderate but depicted developmental impairments (44). It is important to notice that this model mimics one type of environmental risk which is necessary but not sufficient by itself alone to reproduce fully the clinical phenotypes. One may need the combination with genetic risks as well. As such, the mildness of the model is evident and may be responsible for absence of behavioral impairments. Furthermore, behavior evaluation was made at childhood age before adulthood where psychiatric symptoms generally occur following IUGR including social problems, poor cognitive performances, anxiety, and schizophrenia (45). Notice that, due to the variability of the caloric restriction model and according to the high number of different techniques used in this study, number of pups in each group was not large enough to assess possible gender differences but it is point to consider in further experiments.

CONCLUSION

In this study, we show that IUGR induced by moderate undernutrition demonstrated mild cerebral impairments. Cortical synaptogenesis and myelination were potentially delayed. Striatal microstructure showed alterations. The white matter integrity assessed in the external capsule presented abnormal development. Lf supplementation showed beneficial restoration in the cortex, in the striatum microstructure and in the white matter organization of the external capsule. Further Lf showed reduced oxidative stress in ACA interneurons. This model does not fully represent the clinical situation as IUGR newborns often cumulate adverse conditions in addition to IUGR, such as hypoxia-ischemia or infection/inflammation. Nevertheless, oral Lf supplementation showed partial neuroprotection in this CR IUGR model. This is also neuroprotective against hypoxia-ischemia and inflammation in the developing brain, and thus represents a very promising tool to reduce developmental diseases in IUGR and preterm infants.

AUTHOR CONTRIBUTIONS

All authors participated to the design of the study. ES performed behavioral analysis. AT performed IUGR model. CL and J-HC performed immunostaining, protein and gene expression analysis. YvdL performed MRI experiments and analyzed the data. YvdL and CL wrote the manuscript. ES, J-HC, AT, KD, and SS revised the manuscript.

FUNDING

ES received a Swiss Excellence Scholarship for Foreign Scholars to perform the study in SVZ laboratory. This study was supported by the Swiss National Fund N° 33CM30-124101/140334 and the Fondation pour Recherches Médicales, Geneva, the Center for Biomedical Imaging (CIBM) of the UNIL, UNIGE, HUG, CHUV, and EPFL, and the Leenaards and Jeantet Foundations.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00046/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
The handling Editor declared a past collaboration with several of the authors, YvdL and SS.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 25 February 2019
doi: 10.3389/fendo.2019.00096

Impaired Progesterone-Responsiveness of CD11c$^+$ Dendritic Cells Affects the Generation of CD4$^+$ Regulatory T Cells and Is Associated With Intrauterine Growth Restriction in Mice

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 26 October 2018
Accepted: 01 February 2019
Published: 25 February 2019

Citation:
Thiele K, Hierweger AM, Riquelme JIA, Solano ME, Lydon JP and Arck PC (2019) Impaired Progesterone-Responsiveness of CD11c$^+$ Dendritic Cells Affects the Generation of CD4$^+$ Regulatory T Cells and Is Associated With Intrauterine Growth Restriction in Mice. Front. Endocrinol. 10:96. doi: 10.3389/fendo.2019.00096

Up to 10% of pregnancies in Western societies are affected by intrauterine growth restriction (IUGR). IUGR reduces short-term neonatal survival and impairs long-term health of the children. To date, the molecular mechanisms involved in the pathogenesis of IUGR are largely unknown, but the failure to mount an adequate endocrine and immune response during pregnancy has been proposed to facilitate the occurrence of IUGR. A cross talk between the pregnancy hormone progesterone and innate immune cell subsets such as dendritic cells (DCs) is vital to ensure adequate placentation and fetal growth. However, experimental strategies to pinpoint distinct immune cell subsets interacting with progesterone *in vivo* have long been limited. In the present study, we have overcome this limitation by generating a mouse line with a specific deletion of the progesterone receptor (PR) on CD11c$^+$ DCs. We took advantage of the cre/loxP system and assessed reproductive outcome in Balb/c-mated C57Bl/6 PR$^{flox/flox}$CD11c$^{cre/wt}$ females. Balb/c-mated C57Bl/6 PR$^{wt/wt}$CD11c$^{cre/wt}$ females served as controls. In all dams, fetal growth and development, placental function and maternal immune and endocrine adaptation were evaluated at different gestational time points. We observed a significantly reduced fetal weight on gestational day 13.5 and 18.5 in PR$^{flox/flox}$CD11c$^{cre/wt}$ females. While frequencies of uterine CD11c$^+$ cells were similar in both groups, an increased frequency of co-stimulatory molecules was observed on DCs in PR$^{flox/flox}$CD11c$^{cre/wt}$ mice, along with reduced frequencies of CD4$^+$ FoxP3$^+$ and CD8$^+$ CD122$^+$ regulatory T (Treg) cells. Placental histomorphology revealed a skew toward increased junctional zone at the expense of the labyrinth in implantations of PR$^{flox/flox}$CD11c$^{cre/wt}$ females, accompanied by increased plasma progesterone concentrations. Our results support that DCs are highly responsive to

progesterone, subsequently adapting to a tolerogenic phenotype. If such cross talk between progesterone and DCs is impaired, the generation of pregnancy-protective immune cells subsets such as $CD4^+$ and $CD8^+$ Treg cells is reduced, which is associated with poor placentation and IUGR in mice.

Keywords: dendritic cells, IUGR, placenta, progesterone, Tregs

INTRODUCTION

In Western societies, up to 10% of pregnancies are affected by intrauterine growth restriction (IUGR) (1), which is defined as a pathological delay in fetal growth. IUGR reduces short-term survival due to immediate neonatal complications such as asphyxia, hypothermia, hypoglycemia and immunodeficiency (2). Moreover, child development and long-term health is impaired. Thus, IUGR children have an increased risk for metabolic, immunologic and neurodevelopmental impairments (3–5). In addition, clinical studies also reveal an association between IUGR and a significantly higher incidence of metabolic, renal and cardiovascular diseases later in life of the children (6). The molecular mechanisms involved in the pathogenesis of IUGR are largely unknown. However, various pathways have been proposed to contribute to the development of IUGR including maternal, fetal, genetic, and placental factors (2).

In order to ensure adequate placentation and subsequently fetal growth, the maternal immune, and endocrine system needs to actively adapt to the semiallogenic fetus. One key features of this tailored immune response are tolerogenic dendritic cells (tDC) present at the feto-maternal interphase during early pregnancy. The tDCs are characterized by a reduced expression of co-stimulatory molecules and enhanced IL-10 production. Functionally, tDCs promote the generation of regulatory T (Treg) cells (7–9). A number of markers with immunomodulatory potential including Galectin-1 or steroid hormones have been suggested to initiate the generation of tDCs from the less stable subset of immature DCs and sustains their ability to produce IL-10 (8, 10, 11). However, due to technical limitations, it is still unknown which immunomodulatory marker directly triggers the generation of tDCs.

It was the aim of this study to overcome these limitations and address this gap in knowledge. We hereby focussed on the role of progesterone in modulating DC functions by utilizing a cell-specific knockout of the intracellular progesterone receptor (PR) on DCs, as such approach allows to investigate the direct effect of progesterone on dendritic cells and its role in pregnancy outcome.

MATERIALS AND METHODS

Generation of $PR^{flox/flox}CD11c^{cre/wt}$ Mice

$PR^{flox/flox}$ and $CD11c^{cre/wt}$ mice were kindly provided by John P. Lydon from Baylor College of Medicine, Houston, Texas, US and Manuel Friese from University Medical Center Hamburg-Eppendorf, Hamburg, Germany, respectively. In order to generate female mice with a selective KO of the PR on CD11c dendritic cells, $PR^{flox/flox}$ females, and $CD11c^{cre/wt}$ males were mated in the Animal Facility of the University Medical Center Hamburg-Eppendorf (**Figure 1A**). Subsequently, male $PR^{flox/wt}$ $CD11c^{cre/wt}$ and female $PR^{flox/wt}$ $CD11c^{wt/wt}$ offspring were mated in order to generate $PR^{flox/flox}$ $CD11c^{cre/wt}$ and $PR^{wt/wt}$ $CD11c^{cre/wt}$ animals. Experimental mice were obtained by mating male mice with a genotype of $PR^{flox/flox}$ $CD11c^{cre/wt}$ and $PR^{wt/wt}$ $CD11c^{cre/wt}$ with $PR^{flox/flox}$ $CD11c^{wt/wt}$ and $PR^{wt/wt}$ $CD11c^{wt/wt}$ females, respectively. Hence, the cre expression was always transmitted from the father to avoid a premature potential impact of an impaired Progesterone-DC-crosstalk.

Isolation of $CD11c^+$ and $CD11^{neg}$ Cells

In order to confirm the selective KO of the PR on CD11c dendritic cells, the spleen was harvested from WT, $PR^{neg}CD11c$, and $PR^{flox/wt}$ $CD11c^{cre/wt}$ mice. Single cell suspensions were obtained as described previously (12). In brief: the tissue was mashed with the plunger of a sterile disposable syringe in circles through a 40 µm cell strainer (Falcon Cell Strainer 40 µm, BD Bioscience, VWR, Germany). The resulting cell suspension was centrifuged for 8 min with 450 × g at 4°C and the supernatant was discarded. Subsequently, a red cell blood (RBC) lysis was performed using RBC lysis buffer (eBioscience, San Diego, CA) according to the manufacturer's instruction. After centrifugation the cell pellet was resuspended in MACS-Buffer (1xPBS, 0.5% BSA. 2 mM EDTA). $CD11c^+$ cells were enriched by magnetic cell separation using the CD11c MicroBeads UltraPure mouse kit (MACS®, Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instruction. Finally, fluorescence-activated cell sorting of $CD11c^+$ and $CD11^{neg}$ cells was performed to achieve highest purity.

DNA Isolation and Polymerase Chain Reaction (PCR)

DNA was isolated from mouse tail and $CD11c^+$ and $CD11^{neg}$ cells obtained from WT, $PR^{neg}CD11c$ and $PR^{flox/wt}$ $CD11c^{cre/wt}$ mice using the DNeasy Kit (Qiagen) according to the manufacturer's protocol.

PCR analysis was performed as 3-Primer-PCR in 50 µl reactions using the Mastercycler® nexus GX2 (Eppendorf). Primer sequences used were previously published (13) and ordered from TIB Molbiol. The PCR program consisted of initial 94°C for 10 min followed by 30 cycles: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Amplicons were visualized using Agarose gel electrophoresis. Expected band sizes were 226bp for the wildtype allele, 260 bp for the floxed allele and 381 bp for the KO allele.

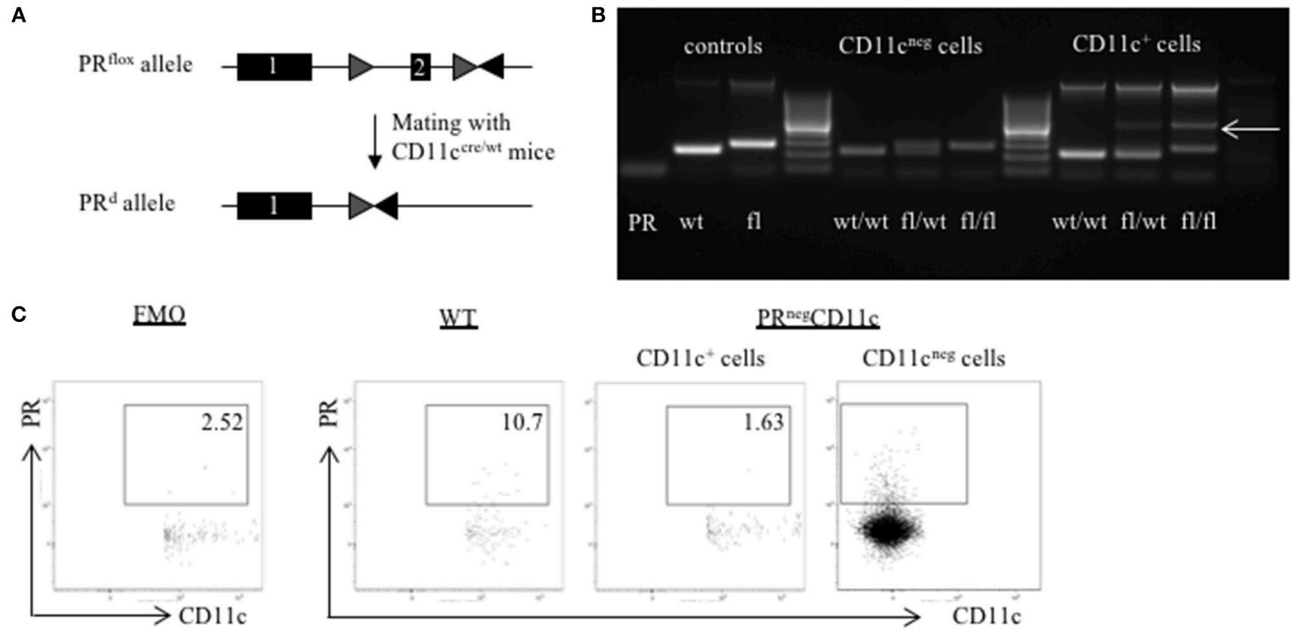

FIGURE 1 | Generation of a selective knockout (KO) of the progesterone receptor (PR) on CD11c dendritic cells (DCs): **(A)** $PR^{flox/flox}$ mice were crossed with $CD11c^{cre/wt}$ transgenic mice resulting in recombination of the PRflox allele to a PR null allele (PRd) on $CD11c^{+}$ cells. **(B)** The selective KO of the PR on $CD11c^{+}$ cells was confirmed on DNA level. PCR amplicons were visualized using agarose gel electrophoresis and band sizes were 226 bp for the wildtype allele, 260 bp for the floxed allele, and 381 bp for the KO allele, respectively. **(C)** The selective KO of the PR on $CD11c^{+}$ DCs harvested from WT and $PR^{neg}CD11c$ mice was confirmed on protein level by means of flow cytometry. The first dot plot on the left side is a fluorescence minus one (FMO) control for the PR antibody.

Timed Pregnancies

Eight to ten weeks old $PR^{flox/flox}CD11c^{cre/wt}$ female mice with a C57Bl/6 background were mated to Balb/c male mice. Aged-matched Balb/c-mated $PR^{wt/wt}CD11c^{cre/wt}$ females served as controls in order to control for unwanted side effects due to the expression of the cre. For simplicity we will refer to $PR^{flox/flox}CD11c^{cre/wt}$ mice as $PR^{neg}CD11c$ and to $PR^{wt/wt}CD11c^{cre/wt}$ control mice as WT.

The presence of a vaginal plug in the morning was considered as gestation day (gd) 0.5. Maternal weight control on gd 8.5 and 10.5 was conducted to confirm pregnancy. Animals were kept under 12 h light/ dark cycles and received food and water *ad libitum*. All experiments were performed in accordance with the animal ethics approval given by the State Authority of Hamburg (ORG_763).

Tissue Harvesting

On gd 13.5 and 18.5, mice were anesthetized by carbon dioxide ventilation, a blood sample was collected by retro bulbar puncture and subsequently mice were sacrificed by cervical dislocation. The abdomen was opened and the uterus-draining lymph node was harvested and kept in PBS on ice. The uterus was removed and stored in HBSS on ice immediately after the fetuses were isolated from the amniotic membranes to determine fetal weight. Placentas were either stored at −20°C in RNAlater (Ambion by Life Technologies GmbH) or embedded in biopsy cassettes and stored in 4% Formaldehyde solution (36.5–38%, Sigma-Aldrich, St, Louis, US) for 24 h before transfer into 1% Formaldehyde solution for long-term storage. Maternal ovaries were also preserved in RNAlater.

Pregnancy Outcome

Number of implantations and abortions was assessed per pregnant female. The abortion rate refers to the number of fetuses resorbed per litter using the following equation: (number of abortions/number of implantations) * 100.

Tissue Processing

Single cell suspensions of maternal lymph nodes and uteri were obtained as described before (14). In brief, maternal lymph nodes were passed through a cell strainer and after centrifugation at 450 g for 8 min at 4°C, the cell pellet was resuspended in PBS. The uterus was enzymatically digested using 200 U/mL hyaluronidase (Sigma-Aldrich), 1 mg/mL collagenase VIII type (Sigma-Aldrich), and 1 mg/mL bovine serum albumin fraction V (Sigma-Aldrich) dissolved in 5 mL HBSS. Subsequently, the uterus was incubated twice for 20 min in a 37°C water bath with agitation. Intermediately, the solution was recovered and filtered through a mesh. The solution was centrifuged at 450 × g for 10 min at 4°C and resuspended PBS.

Number of viable leukocytes in both tissues was obtained by counting the cells using a Neubauer chamber upon adding Trypan Blue stain (0.4 %, Life Technologies GmbH, Darmstadt, Germany).

TABLE 1 | Summary of antibodies used in the present study.

Target antigen	Fluorochrome	Clone	Dilution	Source
anti-CD45	Allophycocyanin (APC)-Cyanine (Cy)7	30-F11	1:400	BD
anti-CD3	R-phycoerythrin (PE) Cy7	145-2C11	1:200	Biolegend
anti-CD8	Brilliant Violet (BV) 650	53-6.7	1:100	Biolegend
anti-CD4	Pacific Blue	RM4-5	1:400	Biolegend
anti-FoxP3	PE	FJK-16s	1:200	eBioscience
anti-CD122	PerCP eFluor 710	TM-b1	1:100	eBioscience
F4/80	BV421	BM8	1:100	Biolegend
anti-CD11c	BV785	N418	1:100	Biolegend
MHCII	APC	M5/144.15.2	1:200	BD
anti-CD80	BV605	16-10A1	1:100	Biolegend
anti-CD86	BV605	GL-1	1:100	Biolegend

Flow Cytometry

For flow cytometric analyses, 1.0×10^6 maternal lymph node and uterus cells were used. Non-specific binding was blocked by rat anti-mouse CD16/CD32 Mouse Fragment crystallizable (Fc) Block (1:200, BD Bioscience) and Normal Rat Serum (1:100, eBioscience) for 15 min at 4°C. Subsequently, the cells were incubated with the respective antibodies for 30 min for surface and intracellular stainings. Antibodies used in this study are displayed in **Table 1**. In case of solely surface staining, 7-amino-actinomycin D (7AAD, 1:400, Biolegend) was used to identify dead cells. For intracellular staining, cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience). Dead/live staining was performed with eFluor 506 viability dye (eBioscience).

In order to quantify the expression of the PR by flow cytometry, cells were stained with the respective surface markers and subsequently permeabilized with Cytofix/Cytoperm (eBioscience) according to the manufacturer's instructions. Afterwards, cells were blocked with mouse serum preventing non-specific binding of antibodies to intracellular proteins and then incubated with Progesterone Receptor Monoclonal Antibody (R.809.9, Invitrogen) for 30 min. A FITC-conjugated secondary antibody (1:200) was added for additional 20 min.

Flow cytometric data were acquired using a BD LSRFortessa II (BD Biosciences) and analyzed using FlowJo (Tree Star, Ashland, OR, USA).

Placenta Histology

Placentas harvested on gd 13.5 and 18.5 were embedded in paraffin. Subsequently, 4 µm thick histological sections were prepared at the mid-sagittal plane using a microtome (SM2010R, Leica, Bensheim, Germany). Slides were dewaxed and rehydrated using xylene and ethanol. Masson-Goldner trichrome staining was performed following standard protocol (15). Subsequently slides were also scanned with a Mirax Midi Slide Scanner. Histomorphological analyses of placental areas were performed by two independent observers using Pannoramic Viewer (3DHistech Kft. Budapest, Hungary).

Progesterone Analysis

Maternal blood samples were centrifuged at 10,000 g for 20 min at 4°C and the supernatant plasma was immediately frozen at −20°C. For progesterone analysis, plasma samples were diluted 1:200 using ELISA Buffer and measured with a competitive immunoassay (Progesterone ELISA Kit, Cayman Chemical, Michigan, USA) on a NanoQuant (Tecan Group AG, Männedorf, Switzerland) according to manufacturer's instructions.

Theiler Scoring of Fetuses on gd13.5

Fetal development of mouse embryos was evaluated by observation of Bouin-fixed fetuses under a Zeiss Stemi 2000-C stereomicroscope according to Theiler's description (16). Main criteria to differentiate the developmental stages at gd 13.5 have been the formation of the pinna, fingers and feet, and the presence or absence of 5 rows of whiskers.

RNA Isolation and cDNA Synthesis

Following tissue harvesting, half of the placentas were preserved in RNAlater at −20°C. Tissue homogenization was carried out using micro packaging vials with ceramic beads (1.4 mm) in a Precellys® 24 Tissue Homogenizer (Peqlab). RNA isolation and DNA digestion were conducted by use of RNeasy Plus Universal Mini Kit (QIAGEN) and DNA-free Kit (Applied Biosystems by Thermo Fisher), respectively. cDNA synthesis was performed with random primers (Invitrogen by Thermo Fisher). Concentration and purity of RNA and cDNA were assessed employing NanoQuant (Tecan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Gene expression analyses of ovarian tissue was performed using gene expression assays (Applied Biosystems by Thermo Fisher) for steroidogenic acute regulatory protein (Star, Mm00441558_m1), 3β-hydroxysteroid dehydrogenase (Hsd3b1, Mm01261921_mH), and 20αHSD (Akr1c18, Mm00506289_m1). Beta-actin (Actb) and ubiquitin C (Ubc) served as endogenous control to normalize cDNA content. Gene expression analyses of placental tissue were carried out using gene expression assays (Applied Biosystems by Thermo Fisher) for the following targets: insulin like growth factor 1 (Igf1, Mm00439560_m1), hydroxysteroid 11-beta dehydrogenase (Hsd11b) 1 and 2 (Mm00476182_m1 and Mm01251104_m1), placental growth factor (Plgf, Mm00435613_m1), epidermal growth factor (Egf, Mm00438696_m1), vascular endothelial growth factor A (Vegf, Mm00437306_m1), B cell leukemia/lymphoma 2 (Bcl2, Mm00477631_m1) and soluble FMS-like tyrosine kinase 1 (sFlt1, Mm00438980_m1), heme oxygenase 1 (Hmox1, Mm00516005_m1), galectin-1 (Gal-1, Mm00839408_g1), and placental lactogen II (Prl3b1, Mm00435852_m1). RNA polymerase II subunit A (Polr2a, Mm00839502_m1) and ubiquitin C (Ubc, Mm02525934_g1) served as endogenous controls (17). All reactions were performed in 50 cycles using

a standard two-step RT-PCR: initial 50°C for 2 min and 95°C for 10 min, 15 s denaturation at 95°C and 60 s annealing and extension at 60°C with the NanoQuant5 Real-Time PCR System (Applied Biosystems) and the corresponding software. The fold change of PR^{neg}CD11c over WT control expression was calculated employing the ΔΔCt method (18).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA, USA). All results are expressed as means ± standard error of the mean (SEM). Means between groups were compared using unpaired *t*-test. Welch's *t*-test was used in case of unequal standard deviation and unequal sample sizes between groups (19).

RESULTS

Confirmation of the Selective Knockout of the PR on $CD11c^+$ DCs

In order to confirm the selective knockout of the PR on $CD11c^+$ DCs on a DNA level, we performed genotyping of tail biopsies and $CD11c^+$ and $CD11^{neg}$ cells of WT, $PR^{flox/wt}CD11c^{cre/wt}$ and PR^{neg}CD11c mice, respectively. The agarose gel shows the amplicons of tail biopsies from WT mice at the expected band size of 226 bp and from PR^{neg}CD11c at 260 bp. PCR of sorted $CD11c^{neg}$ spleen cells from the respective genotypes resulted also in a 226 bp amplicon for WT and in a 260 bp amplicon for PR^{neg}CD11c while cells from heterozygote mice showed both bands. Equally, PCR of sorted $CD11c^+$ spleen cells from WT mice resulted in a 226 pb amplicon. $CD11c^+$ spleen cells from $PR^{flox/wt}CD11c^{cre/wt}$ and PR^{neg}CD11c animals exhibit a band of 381 bp for the KO allele (**Figure 1B**). In order to confirm this selective KO also on the protein level, we used a monoclonal antibody against the PR for detection by flow cytometry. $CD11c^+$ cells harvested from PR^{neg}CD11c mice showed a lower frequency of the PR on DC compared to cells isolated from WT mice (**Figure 1C**). A positive PR expression could be confirmed on $CD11c^{neg}$ cells from PR^{neg}CD11c mice. However, as we also detected $CD11c^{pos}$ PR^{pos} cells at low frequencies among cells isolated from PR^{neg}CD11c mice, unspecific binding of the secondary antibody must be assumed. Alternatively, the cre recombinase may not exhibit 100% efficiency.

Impaired Progesterone-Responsiveness of $CD11c^+$ DCs Affects Fetal Growth

Pregnancy rate did not significantly differ between WT and PRnegCD11c female mice (**Figure 2A**). Pregnancy outcome was assessed on gd 13.5 as well as 18.5. We could not identify any modification with regards to number of implantations (**Figures 2B,E**) and abortion rate (**Figures 2C,F**) in litters from WT and PR^{neg}CD11c females. However, we observed a significantly reduced fetal weight on gestational day 13.5 and 18.5 in litters of PR^{neg}CD11c females (**Figures 2D,G**) along with a minor tendency toward a decreased placental weight on gd 18.5 (**Figure 2H**). We did not observe sex-specific effects, as a similar fetal weight could be observed in male and female offspring (**Figure 2J**). Further, the chosen mating combination yields to two fetal genotypes: $PR^{flox/wt}CD11c^{cre/wt}$ and $PR^{flox/wt}CD11c^{wt/wt}$. In order to exclude an impact of the fetal expression of the cre recombinase on the fetal weight, we assessed the fetal genotype from 2 litters and could exclude that the fetal genotype caused alterations of the fetal weight (**Figure 2K**). Using Theiler staging as a criterion to assess fetal development, we observed only a minor delay in fetuses from PR^{neg}CD11c mother (**Figure 2L**). A representative photographic image of fetuses from WT and PR^{neg}CD11c mice in shown in **Figure 2M**.

Impaired Progesterone-Responsiveness of $CD11c^+$ DCs Affects Maternal Immune Adaptation

Flow cytometry analysis from the uterus of gd 13.5 revealed similar frequencies of uterine $CD11c^+$ cells in both groups (**Figure 3A**). While the co-expression of MHCII was not different between groups (data not shown), we observed increased frequencies of DCs expression co-stimulatory molecules CD80 or CD86 in PR^{neg}CD11c mice (**Figure 3B**). Further, we identified reduced frequencies of $CD4^+$ $FoxP3^+$ and $CD8^+$ $CD122^+$ regulatory T (Treg) cells in uteri of PR^{neg}CD11c dams (**Figures 3C,D**). Representative dot plots are shown in **Figures 3E–G**. We made similar observations of unaltered CD11c frequencies and increased co-expression of CD80 and CD86 in cells isolated from uterus-draining lymph nodes (**Figures 3H,J**), whereas no significant differences were detectable for $CD4^+$ $FoxP3^+$ Treg cell frequencies (**Figure 3K**) and $CD8^+$ $CD122^+$ Treg cells (**Figure 3L**) between WT and PR^{neg}CD11c dams.

Placental Histomorphology and Plasma Progesterone Levels Was Modulated on gd 13.5

Placenta morphology was assessed on gd 13.5 and 18.5 by Masson-Goldner trichrome staining on mid-sagittal sections. The overall placental surface area did not differ between groups (**Figures 4A,E**). However, a skew toward an increased junctional zone at the expense of the labyrinth could be detected in PR^{neg}CD11c females compared to WT females on gd 13.5 (**Figures 4B,C**), which resulted in a significantly decreased placental ratio (labyrinth/junctional zone, **Figure 4D**), a proxy for placental function (20). The same observation could be made when analyzing the placentas from gd 18.5, but it did not reach statistical significance (**Figures 4F–H**). Representative photomicrographs from gd 13.5 and 18.5 placentas are shown in **Figure 4J**.

We also determined plasma progesterone concentrations and observed a significant increase in PR^{neg}CD11c mice compared to WT controls on gd 13.5 (**Figure 5A**). Subsequent qPCR analysis of maternal ovaries on gd 13.5 revealed no differences in gene expression of the ovarian steroidogenic acute regulatory protein (Star) (**Figure 5B**) and 3β-hydroxysteroid dehydrogenase (Hsd3b1), which converts pregnenolone to progesterone (**Figure 5C**). Progesterone metabolism was also not modulated in the KO line, suggested by the low, unaltered

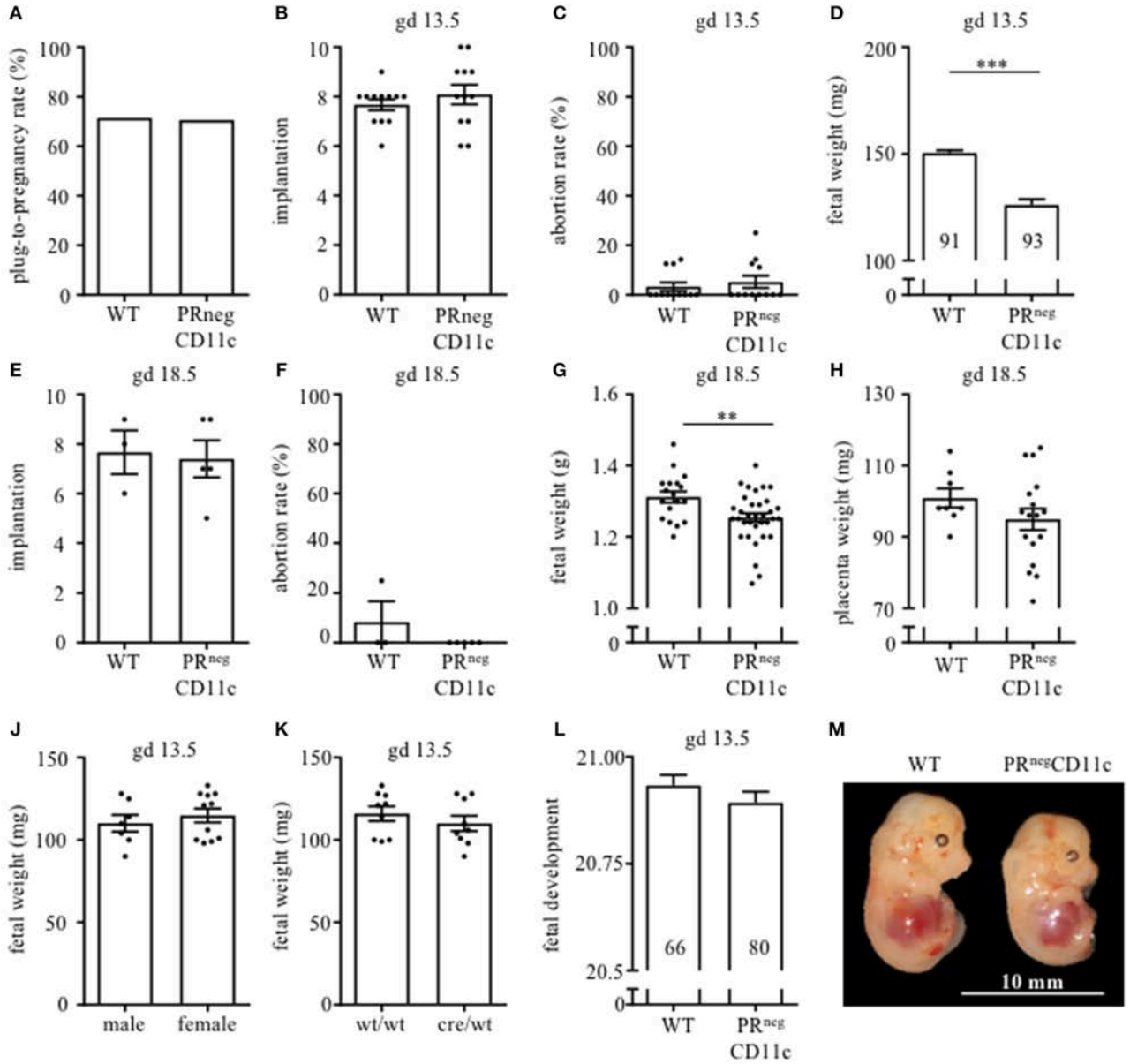

FIGURE 2 | Impaired progesterone-responsiveness of CD11c^{+} dendritic cells affects fetal growth: WT and PR^{neg}CD11c female mice were allogenically mated to Balb/c males and pregnancy outcome was assessed, maternal phenotype is always indicated below: **(A)** Plug-to-pregnancy rate. On gestational day (gd) 13.5, we assessed number implantation **(B)**, abortion rate **(C)** and fetal weight **(D)**. For gd 18.5, number implantation **(E)**, abortion rate **(F)**, fetal weight **(G),** and placental weight **(H)** were determined. Fetal weight of fetuses from PR^{neg}CD11c dams is further shown dependent on fetal sex **(J)** and fetal genotype **(K)**. **(L)** Fetal development was scored on gd13.5 according to Theiler criteria and a representative picture from gd 13.5 fetuses of WT (left) and PR^{neg}CD11c (right) mice is shown in **(M)**, white line in the picture denote 10 mm. Data are represented as mean ± SEM. ***$p \leq 0.001$, **$p \leq 0.01$.

ovarian expression of 20α-hydroxysteroid dehydrogenase (Akr1c18) (**Figure 5D**).

We also analyzed the differential expression of placental genes that has been linked to the pathogenesis of IUGR (**Figure 5E**) and could demonstrate that placental growth factor (Plgf) was significantly increased in placentas from PR^{neg}CD11c mothers. In contrast, significantly decreased placental expression was observed for epidermal growth factor (Egf), vascular endothelial growth factor A (Vegfa) and insulin like growth factor 1 (Igf1). In addition, hydroxysteroid 11-beta dehydrogenase 2 (Hsd11b2) and placental lactogen II (Prl3b1) showed a trend toward reduced expression in PR^{neg}CD11c placentas, but did not reach levels of significance. B cell leukemia/lymphoma 2 (Bcl-2), soluble FMS-like tyrosine kinase 1 (sFlt1), heme oxygenase 1 (Hmox), and galectin-1 (Gal-1) were not affected by the selective KO of the PR.

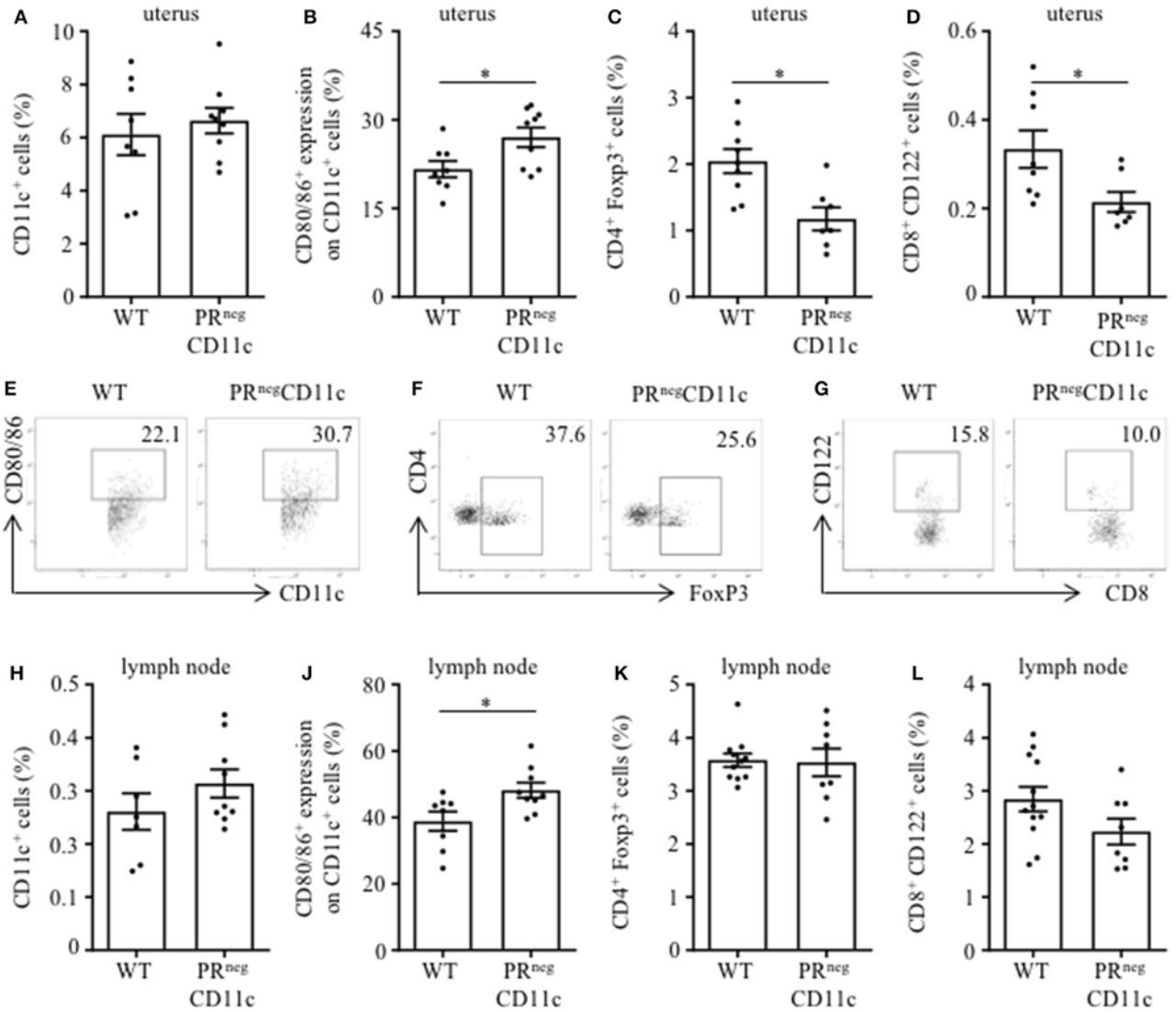

FIGURE 3 | Impaired progesterone-responsiveness of CD11c$^+$ dendritic cells (DCs) affects maternal immune adaptation: WT and PR^{neg}CD11c female mice were allogenically mated and flow cytometric analysis was performed on gestation day 13.5. Graphs present the frequencies of **(A)** CD11c$^+$ DCs, **(B)** the co-expression of CD80 and CD86, **(C)** CD4$^+$FoxP3$^+$ Treg cells, and **(D)** CD8$^+$CD122$^+$ T cells in uteri harvested from WT and PR^{neg}CD11c dams. Representative dot plots display CD80/86 expression on CD11c$^+$ cells **(E)**, FoxP3$^+$ expression on CD4$^+$ cells **(F),** and CD122$^+$ expression in CD8$^+$ T cells **(G)** of WT and PR^{neg}CD11c mice. Respective cell frequencies in the right corner are expressed as percentage of CD11c$^+$, CD4$^+$ and CD8$^+$ cells, respectively. **(H–L)** Flow cytometric analysis of CD11c$^+$ DCs **(H)**, the co-expression of CD80 and CD86 **(J)**, CD4$^+$FoxP3$^+$ Treg cells **(K),** and CD8$^+$CD122$^+$ T cells **(L)** in uterus-draining lymph node harvested from WT and PR^{neg}CD11c dams. Bars represent mean ± SEM. *$p \leq 0.05$, unless otherwise stated, cell frequencies are expressed as percentage of living CD45$^+$ cells.

DISCUSSION

The steroid hormone progesterone is known to be indispensable for successful reproduction, as demonstrated in mice carrying a null mutation of the PR gene (PR-KO mice) (21). Here, male mice do not show any abnormalities, but female mice display significant functional defects in all reproductive tissues, including the inability to ovulate, uterine hyperplasia and inflammation and severely limited mammary gland development (21).

Due to the complexity of endocrine-immune cross talk particularly in the context of steroid actions, the generation of a mouse model enabling conditional excision of PR function (PRflox mice) in a cell- or tissue-specific manner provides a useful tool to study progesterone-dependent pathways and processes (13). Using such approach in the present study, we observed that mice devoid of PR on DCs do not exhibit major reproductive abnormalities, such as an altered plug-to-pregnancy rate or increased fetal loss rate. However, the offspring of such PR^{neg}CD11c dams were severely affected by IUGR, as fetal weight was ∼15% lower compared to WT dams on gd 13.5. This result was not compromised by possible alterations of the viable litter size, by fetal sex or fetal genotype as such effects were not

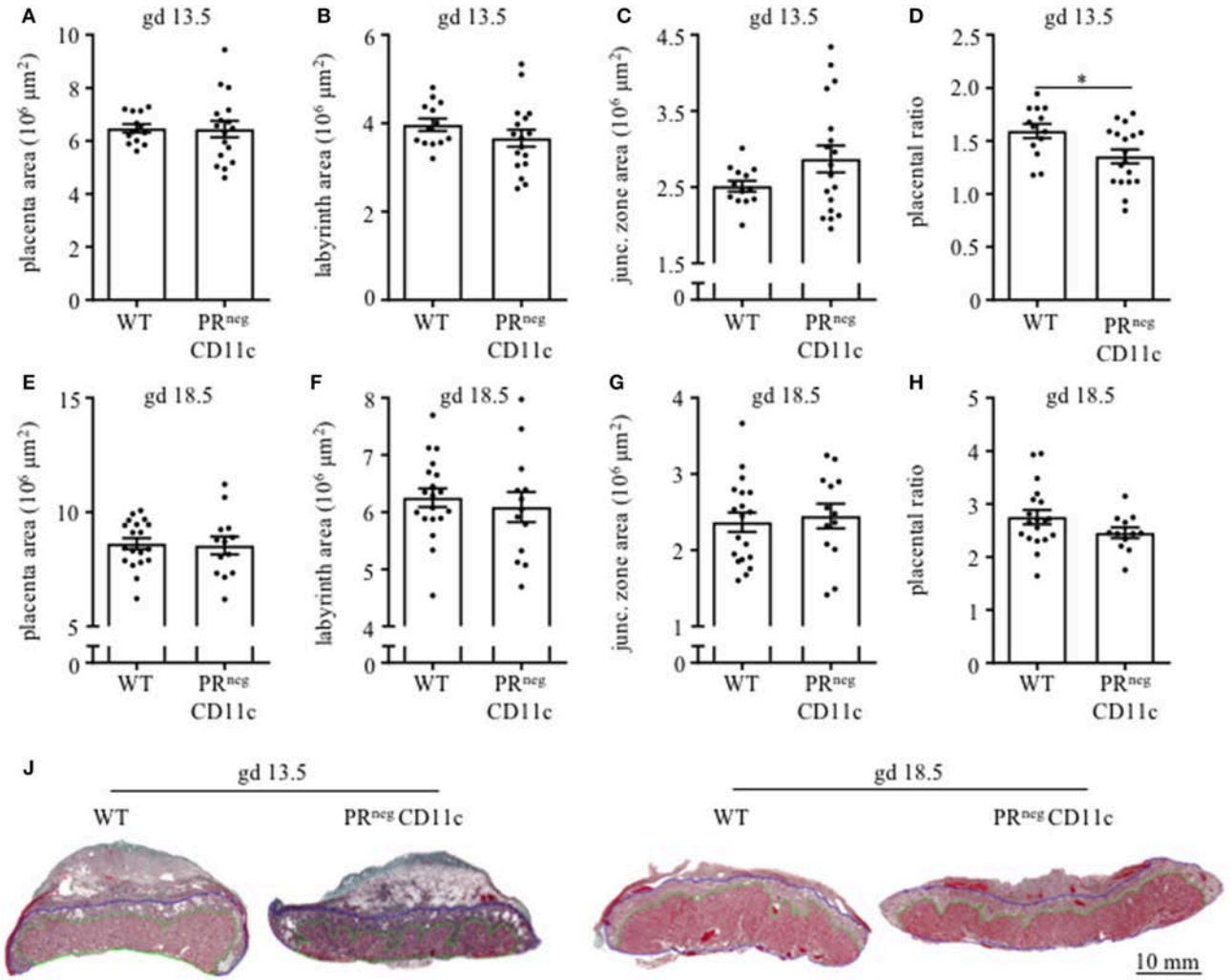

FIGURE 4 | Impaired progesterone-responsiveness of CD11c$^+$ dendritic cells affects placental morphology: WT and PR^{neg}CD11c female mice were allogenically mated and placentas harvested on gestation day (gd) 13.5 und 18.5 were evaluated by Masson-Goldner trichrome staining allowing the differentiation of the labyrinth and junctional zone. **(A,E)** show total placenta area on gd 13.5 and 18.5, respectively. Area of the labyrinth **(B,F)** and the junctional zone **(C,G)** have been assessed and the placental ratio **(D,H)** was calculated. **(J)** Representative photomicrographs illustrating mid-sagittal sections of gd 13.5 and 18.5 placental tissue from WT (left) and PR^{neg}CD11c (right) mothers, black line in the picture denotes 10 mm, green lines encircle the labyrinth, blue lines surround the junctional zone. Data are represented as mean ± SEM. *$p \leq 0.05$.

detectable between the groups and could therefore be excluded as potential confounder. A graphical summary of the main finding is presented in **Figure 6**.

Our data further provide strong evidence that progesterone is directly involved in arresting DCs in a tolerogenic phenotype, mirrored by the increased expression of the co-stimulatory molecules CD80/CD86 in mice where the PR is lacking on DCs. This sheds new light on the recent recognition that progesterone promotes tolerance of the adaptive immune system during gestation via glucocorticoid receptor-dependent pathways (22) and suggests that progesterone modulates innate immune response during gestation through the PR by inducing a tolerogenic phenotype in DCs. However, the extrapolation of cellular functions solely based on the expression of cell surface markers is limited. Thus, the presumed tolerogenic function of DCs during pregnancy—or lack thereof in the absence of the PR—requires confirmation upon functional in-depth characterization in future experiments.

Such tDC may then account for the generation of CD4$^+$ Treg cells, which has been suggested by published data (7–9, 23, 24). Our data support that progesterone considerably induces the generation of CD4$^+$ Treg cells via DC-dependent pathways rather than direct effects on CD4$^+$ T cells. However, in order to prove this causality, it will be necessary to perform adoptive transfer experiments demonstrating that replenishing Tregs in mice devoid of the PR on CD11c cells will prevent the development of the observed IUGR. Similarly, we provide evidence that the generation of a newly identified CD8$^+$

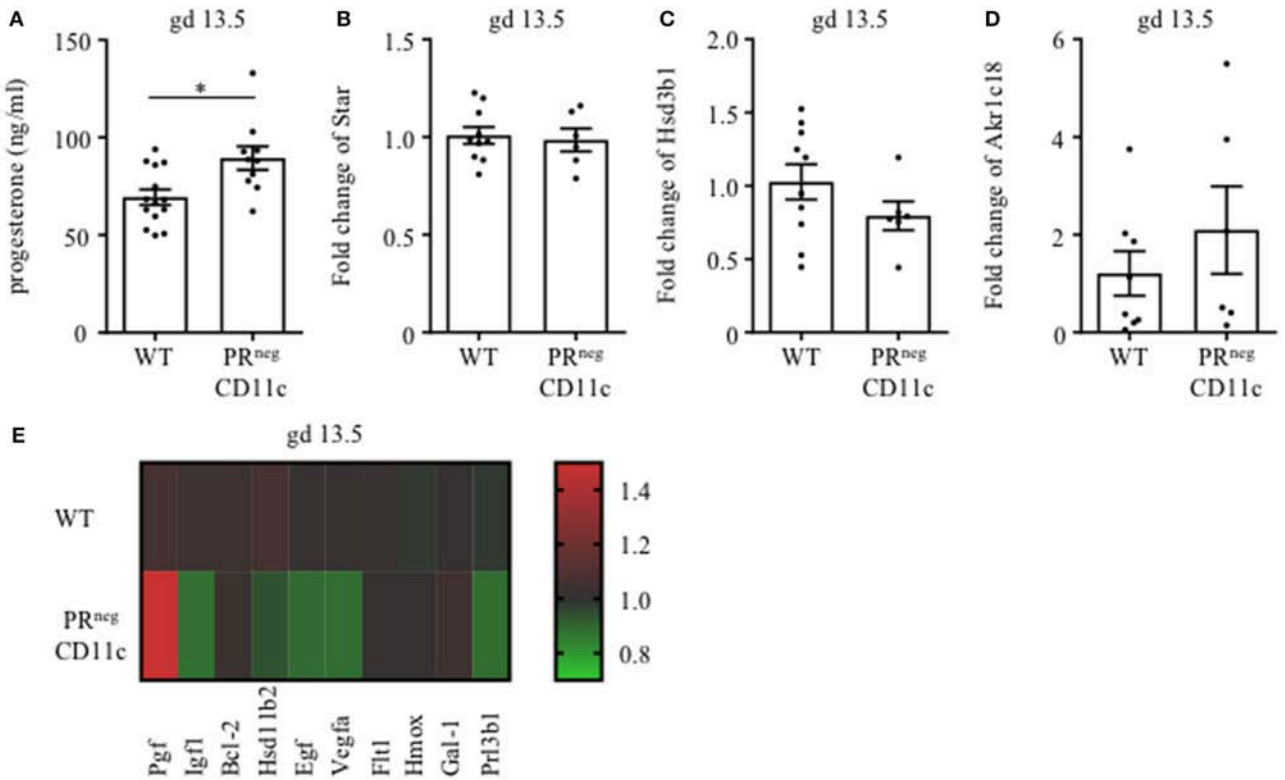

FIGURE 5 | Selective knockout of the progesterone receptor (PR) on CD11c$^+$ dendritic cells affects gene expression analysis of ovarian and placental tissue: **(A)** Plasma progesterone levels of WT and PR^{neg}CD11c female mice on gd 13.5 as analyzed by ELISA. **(B–D)** Ovarian expression of steroidogenic acute regulatory protein (Star), 3β-hydroxysteroid dehydrogenase (Hsd3b1), and 20αHSD (Akr1c18) was assessed by qPCR from tissue harvested on gd 13.5. The fold change over WT control expression was calculated using beta-actin (Actb) and ubiquitin C (Ubc) as endogenous control and employing the ΔΔCt method. **(E)** Heatmap summarizing the placental expression of placental growth factor (Plgf), insulin like growth factor 1 (Igf1), B cell leukemia/lymphoma 2 (Bcl2), hydroxysteroid 11-beta dehydrogenase (Hsd11b2), epidermal growth factor (Egf), vascular endothelial growth factor A (Vegfa), soluble FMS-like tyrosine kinase 1 (sFlt1), heme oxygenase 1 (Hmox1), Galectin-1 (Gal-1), and placental lactogen II (Prl3b1) calculated by qPCR from gd 13.5 placentas. The fold change over WT control expression was calculated using RNA polymerase II subunit A (Polr2a) and Ubc as endogenous control and employing the ΔΔCt method. Bars represent mean ± SEM. *$p \leq 0.05$.

CD122$^+$ Treg cell subset involved in promoting fetal growth (15) is also critically dependent on the possibility of DCs to respond to progesterone. It has been suggested that progesterone upregulates placental Hmox1 expression, hereby promoting the generation of CD8$^+$ CD122$^+$ T cells. However, since placental expression of Hmox1 was not altered in mice lacking the PR on DCs, we propose that placental Hmox1 as well as tDCs may independently promote the generation of CD8$^+$ CD122$^+$ T cells.

As CD11c cells are involved in a variety of functions, e.g., thymic T cell development, one might suspect that the missing PR on CD11c cells may have an impact on the adaptive immune response even outside the context of pregnancy, but progesterone levels are rather low in pre-adolescent female mice and also during the menstrual cycle. Further, reduced CD4$^+$ Treg frequencies were exclusively observed locally in the uterus of PRnegCD11c mice, whereas CD4$^+$ Treg frequencies in secondary lymphoid organs or peripherally were unaffected. Hence, an effect of the missing PR on CD11c cells on the adaptive immune system in a non-pregnant state can largely be excluded.

We could pinpoint the cause for IUGR to the lack of the PR on DCs in the mother rather than the fetal genotype, as modulation was not dependent on the fetal genotype and only affected by the maternal PR expression on DCs. Modulation of fetal weight loss can result from placental insufficiency, a known contributor to IUGR (25). We could confirm such effect, as poor placental development was present in litters of PR^{neg}CD11c dams. Whilst placental size and weight was unaffected by the maternal genotype, the junctional zone was increased at the expense of the labyrinth at implantation sites of PR^{neg}CD11c dams. Such skew of placental functional areas might impair fetal supply with nutrients and oxygen (26).

Normally, impaired fetal growth and poor placentation is accompanied by reduced progesterone levels, as seen in response to prenatal challenges (14, 15). Hence, we were rather intrigued by the high progesterone levels in PR^{neg}CD11c dams. This overproduction of progesterone, which appears to be an attempt to compensate for its failure to interact with DC, may lead to other placental modifications contributing to the development of IUGR, such as placental vasculogenesis and angiogenesis. Both are modulated by endothelium-specific molecules such as PlGF and VEGF (27, 28) which compete for the same cell surface tyrosine kinase receptors, VEGFR-1/Flt-1, but

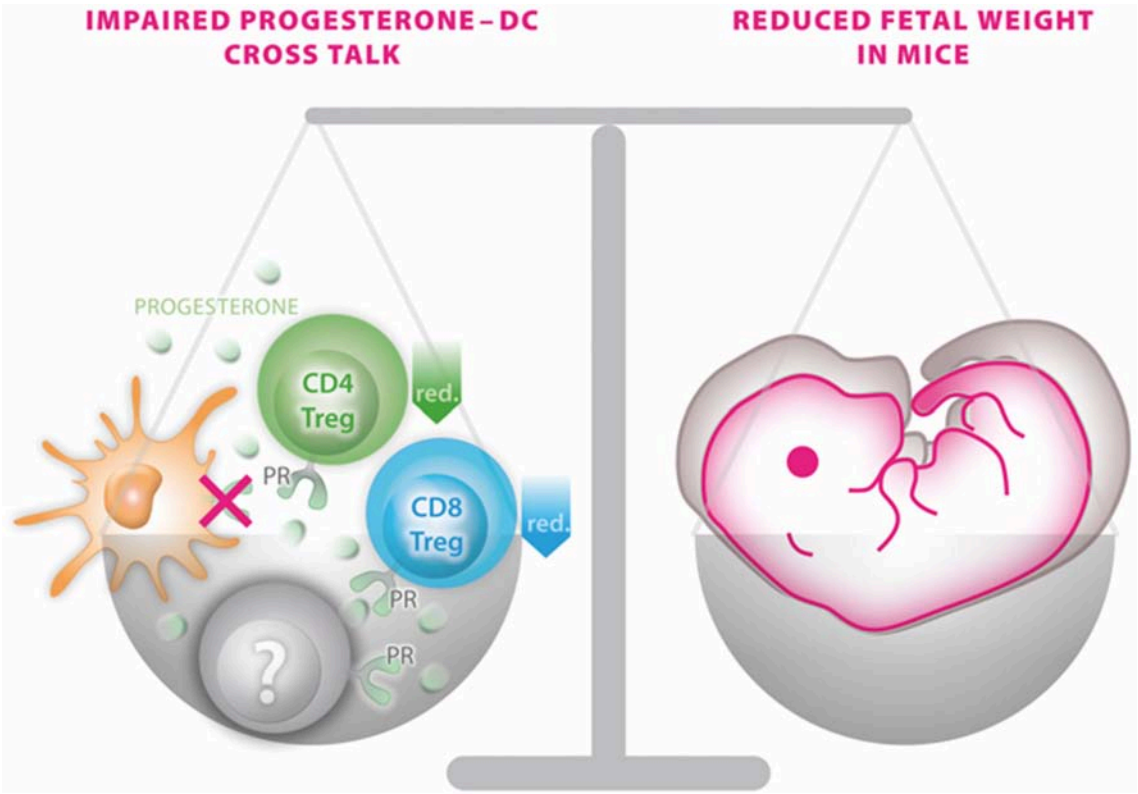

FIGURE 6 | Graphical scenario depicting the impact of the selective knockout of the progesterone receptor on CD11c^{+} dendritic cells (DCs) and its consequences for fetal growth. An impaired progesterone-DC cross talk disturbs the fine-tuned balance of a functional immune response of DCs to progesterone by affecting CD4 and CD8 Treg populations (and potentially also on yet to be identified immune cells marked in gray) on one site and fetal outcome on the other side.

differentially induce angiogenesis; VEGF controls branching of blood vessels, while PlGF promotes a low resistance vascular network during mid- to late pregnancy (28). However, both factors are sensitive for changes in the microenvironment. As progesterone release is reduced in hypoxic condition (29), it could be speculated that high progesterone favors a hyperoxic environment. In this case, placental PlGF is increased while VEGF is decreased (30), similar to the gene expression we observed in our study. Consequently, superfluous progesterone could result in the decreased vascular branching, along with an increase in fetoplacental flow impedance via differential modulation of PlGF and VEGF (31). Moreover, the decrease of VEGF we observed in dams lacking the PR on DCs is even more striking as the competition for the same receptor should normally increase the quantity of VEGF then acting via the VEGFR-2 which is a major mediator of angiogenic responses (32). On the other hand, there are many studies postulating a decrease of PlGF in IUGR placenta (33–35). Hence, one might speculate that Plgf expression is not dependent on a functional PR-DC crosstalk and the observed over-expression is initiated to support placentation and angiogenesis. An altered placental gene expression in IUGR was reported for EGF (36), which we could also observe in placentas from PR^{neg}CD11c females. Hence, EGF may be a target that requires a communication between progesterone and DCs and a lack of this communication impairs placental vascularization and consequently fetal nourishment. Additionally, IGF1 is known to facilitate the transport of glucose and amino acids across the placenta to the fetus and placental expression is reduced in IUGR and SGA fetuses associated with DNA methylation alterations (37). Our results support that the reduced placental Igf-1 expression observed in PR^{neg}CD11c dams contribute to development of IUGR (38, 39). Taken together, these qPCR results pinpoint the need to characterize the mechanisms underlying placental vascularization in PR^{neg}CD11c mice in future studies.

One limitation of the mouse model we here describe is that we chose CD11c-cre mice to target gene expression in DC (40). Although CD11c is widely accepted as a pan marker for conventional DCs (cDCs), CD11c is also expressed on macrophages and monocytes (41), plasmacytoid DCs and marginally on some lymphocyte subsets (42). Therefore, the CD11c cell specific deletion of the PR we here describe is not fully DC specific. A better option may be the recently generated cDC-restricted cre mouse (zDCcre) using a zinc finger transcription factor, zDC (Zbtb46, Btbd4), which is not expressed by monocytes, pDCs, or other immune cell populations (43, 44). These zDCcre mice could be used in future investigation to knock out the PR in cDCs in order to verify and compare to the present

results. Nevertheless, as the large majority of $CD11c^+$ cells are cDCs, our results provide strong evidence of a critical crosstalk between progesterone and DCs to ensure reproductive success.

Further, the cre-lox system itself shows certain limitations, such as the efficiency of target gene deletion, cre-mediated toxicity and undesired deletions (45). In order to avoid the two latter confounding factors, we used $PR^{wt/wt}CD11c^{cre/wt}$ mice as controls. Even if the CD11c-cre exhibits unwanted side effects, we can exclude an impact on our result, as both groups share the same potential cre-mediated toxicity burden. Upon detection of the PR gene on DNA and protein level, we observe an incomplete gene deletion on DCs. This may result from cell contamination during MACS and sorting, unspecific binding of the secondary antibody, but also be due to a minor deficiency of PR gene target deletion on $CD11c^+$ cells. Although PR deletion might be incomplete, this mouse model has proven to be a suitable tool to investigate progesterone-dependent pathways in DCs.

In summary, we utilized the Cre-Lox recombination to successfully generate a DC-specific knockout of the PR in mice. We could demonstrate that a progesterone-responsiveness of dendritic cells is critical for fetal growth and facilitates the generation of pregnancy-protective $CD4^+$ and $CD8^+$ Tregs and adequate placentation. An association between diseases of insufficient placentation and the maturity of DCs have also been demonstrated in human placenta (46). In addition, human pregnancies complicated by IUGR were shown to exhibit peripheral blood DCs with an altered state of activation (47). These findings highlight the importance that an in-depth understanding of modulators that yield to such DC alterations must be gained, as addressed in the present manuscript.

AUTHOR CONTRIBUTIONS

KT and PA: conceptualization; KT, AH, JL, and MS: methodology; KT, AH, and JR: Investigation; KT, JL, and PA: resources; KT and PA: writing—original draft; All authors: comments on manuscript.

FUNDING

This work was supported by research grants provided by the German Research Foundation to KT (TH 2126/1-1) and PA (KFO296, AR232/26-2, and AR232/27-1).

ACKNOWLEDGMENTS

We would like to thank JL, Francesco J. DeMayo and Manuel A. Friese for providing the $PR^{flox/flox}$ and $CD11c^{cre/wt}$ mice, respectively. We also thank Agnes Wieczorek, Christopher Urbschat, and Thomas Andreas (Department of Obstetrics and Fetal Medicine, University Medical Center Hamburg) for their technical assistance. The flow cytometers used in this study were provided by the FACS Core Unit of the University Medical Center Hamburg-Eppendorf.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 01 March 2019
doi: 10.3389/fendo.2019.00098

Molecular Principles of Intrauterine Growth Restriction in Plasmodium Falciparum Infection

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Malaria in pregnancy still constitutes a particular medical challenge in tropical and subtropical regions. Of the five *Plasmodium* species that are pathogenic to humans, infection with *Plasmodium falciparum* leads to fulminant progression of the disease with massive impact on pregnancy. Severe anemia of the mother, miscarriage, stillbirth, preterm delivery and intrauterine growth restriction (IUGR) with reduced birth weight are frequent complications that lead to more than 10,000 maternal and 200,000 perinatal deaths annually in sub-Saharan Africa alone. *P. falciparum* can adhere to the placenta via the expression of the surface antigen VAR2CSA, which leads to sequestration of infected erythrocytes in the intervillous space. This process induces a placental inflammation with involvement of immune cells and humoral factors. Especially, monocytes get activated and change the release of soluble mediators, including a variety of cytokines. This proinflammatory environment contributes to disorders of angiogenesis, blood flow, autophagy, and nutrient transport in the placenta and erythropoiesis. Collectively, they impair placental functions and, consequently, fetal growth. The discovery that women in endemic regions develop a certain immunity against VAR2CSA-expressing parasites with increasing number of pregnancies has redefined the understanding of malaria in pregnancy and offers strategies for the development of vaccines. The following review gives an overview of molecular processes in *P. falciparum* infection in pregnancy which may be involved in the development of IUGR.

Keywords: malaria, plasmodium, pregnancy, placenta, intrauterine growth restriction, small for gestational age, anemia

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *10 November 2018*
Accepted: *01 February 2019*
Published: *01 March 2019*

Citation:
Seitz J, Morales-Prieto DM, Favaro RR, Schneider H and Markert UR (2019) Molecular Principles of Intrauterine Growth Restriction in Plasmodium Falciparum Infection. Front. Endocrinol. 10:98. doi: 10.3389/fendo.2019.00098

INTRODUCTION

Pregnant women are more susceptible to infection with *Plasmodium falciparum* and present a more severe form of the disease than non-pregnant women (1). The probability of suffering from severe malaria infections is three times higher—with a mortality rate of up to 50% (2, 3). Other complications involve severe anemia, cerebral malaria, and massive pregnancy disorders (4). The increased susceptibility is attributed to two main factors: firstly, physiological processes during pregnancy, such as the altered hormone constellation with suppression of certain immune reactions, and increased body temperature, which makes pregnant women more attractive to Anopheles mosquitoes (5, 6); secondly, the sequestration of *P. falciparum*-infected erythrocytes in the placenta (7).

In placental malaria, *P. falciparum* expresses a special Plasmodium falciparum erythrocyte membrane protein 1 (PfEMP-1), the VAR2CSA antigen, which can bind to chondroitin sulfate A (CSA) produced by trophoblast cells. This interaction promotes the retention of parasites in the intervillous space triggering an inflammatory reaction known as intervillitis. Women in endemic regions often have developed humoral immunity reflected by the production of antibodies against different PfEMP-1 expressing *P. falciparum* strains. However, as the VAR2CSA appears only in pregnancy, primiparous women have no antibodies against this antigen yet, and again, are at high risk for a new *P. falciparum* infection. Infections in further pregnancies are usually less severe due to previous contact with VAR2CSA-expressing *P. falciparum* strains and increasing immunity to VAR2CSA (8). By accumulation in the placenta, *P. falciparum* also evades elimination processes in the spleen. In endemic regions, the peripheral infection can be controlled mostly with acquired partial immunity against *P. falciparum*, while the plasmodia may persist unrecognized in the placenta and can cause maternal anemia as well as fetal developmental disorders (9). In addition to *P. falciparum*, *P. vivax* can also lead to pregnancy complications. However, the consequences are generally less severe and are not presented in this review (4, 10).

Various measures and antimalarials can be taken to prevent and treat malaria during pregnancy but there is a lack of information regarding their safety, efficacy and pharmacokinetics. In all regions affected by malaria, early diagnosis and treatment as well as the use of ITNs (insecticide-treated nets) are crucial. In regions with endemic malaria, intermittent preventive treatment (IPTp) starting at the second trimester with the antimalarial drug sulfadoxine-pyrimethamine is additionally recommended for pregnant women by the World Health Organization (11). A metadata analysis confirmed that this therapy reduces the risk of low birth weight (LBW) when 3 or more doses are administrated, compared to the standard 2-doses regimen (12), but among the estimated 35 million pregnant women eligible for IPTp therapy, in 2017, <50% received two, and only ∼22% received three or more doses of IPTp (11). Further efforts are needed to improve the coverage and access to IPTp for this vulnerable population.

Currently, the most effective first-line treatment recommended by the WHO for the general population is an artemisin-based combination therapy (11). This therapy resulted embryotoxic in animal studies. Therefore, less efficient and less well-tolerated medicines such us quinine and clindamycin have been recommended for women in first trimester pregnancy (13). However, the embryo exposure and toxic effects to artemisins may be different or lower in humans due to their specific placenta morphology (14). This may be supported by growing evidence that artemisins in first trimester pregnancy do not increase the risk of miscarriage, stillbirth or malformations when compared to quinine-based treatment (13, 14). For these reasons a clear conclusion of the risk/benefit ratio of antimalarials medicines cannot be drawn until further studies on pharmacokinetics and safety in humans will be conducted.

Finally, an additional approach to prevent malaria is based on current studies focused on the identification of the most immunogenic epitopes of the VAR2CSA antigen for vaccine development against placental malaria in pregnancy (see below) (15).

P. FALCIPARUM INFECTION IN PREGNANCY

It has been estimated that, in 2007, out of 85.3 million pregnant women in areas at risk for *P. falciparum* malaria, about 2/3 lived in regions with stable (endemic) malaria (16). In these areas, one out of four women at delivery had evidence of placental infection (4). In sub-Saharan Africa alone, malaria was responsible for more than 10,000 maternal and 200,000 perinatal deaths annually until 2009 (2). In 2015, it has been estimated that 900,000 newborns suffer from reduced birth weight due to placental malaria (17). The exact incidence of *P. falciparum* malaria in pregnancy and the resulting IUGR cases remain unknown.

During pregnancy, the clinical appearance of a *P. falciparum* infection depends largely on the maternal immune status, which in turn is associated with the geographical region. If the woman has already acquired immunity, which is usually the case in areas with stable malaria, the infection is often asymptomatic. High fever and complications such as cerebral malaria, hypoglycemia, or pulmonary edema are rare. However, especially in their first pregnancy, women are not protected against placental infection. As the infection may persist undetected, it can lead to pronounced maternal anemia and serious consequences for the unborn child.

Low birth weight (defined as birth weight <2,500 g) is the largest risk factor for infant and child mortality in Africa (18). Independently of malaria infection, growth restricted babies have nine times higher risk of dying within the first month of life than normal weight newborns (19). Annually, around 100,000 children die in Africa as result of malaria-associated LBW (19). The major causes are premature birth and IUGR (20).

It is still unclear which period of infection during pregnancy is the most detrimental for fetal growth. In a recent study including 1190 pregnant women in Burkina Faso, maternal infection after the sixth month of pregnancy was significantly related to higher risk of LBW, while only a trend was found between early infection (≤4 months of pregnancy) and LBW (21). Conversely, a second study reported higher risk of LBW after infection in the second compared to third trimester or at delivery (22). And in a Benin cohort, only infection in early pregnancy was associated with LBW and maternal anemia at delivery (23). Remarkably, a high number of infections during pregnancy leads to an increased risk of LBW independently of the timing (22, 24). As the maternal immunological reactions change during pregnancy, more studies are needed to fully understand the interaction between infection and host response depending on the stage of pregnancy, and how this impare fetal growth.

Since severe maternal anemia and placental malaria are the major mechanisms responsible for malaria-related IUGR, they will be comprehensively addressed in the following sections.

Maternal Anemia in Malaria

Severe anemia (defined as hemoglobin concentration Hb < 7 g/dl) is the main complication of maternal *P. falciparum* infections in regions with stable malaria. In the sub-Saharan region, between 200,000 and 500,000 pregnant women are estimated to develop malaria-associated severe anemia (25–27). Severe anemia can lead to death even if only slight blood loss occurs during delivery. Circulation problems with an increased risk of heart failure and pulmonary edema may also occur (2, 9). In addition to malaria, other causes such as iron, vitamins (e.g., folate, vitamin A, and vitamin B-12) and trace element deficiency or worm infections may further contribute to the occurrence of anemia (28–30).

The pathogenesis of anemia in malaria is generally multifactorial, even in non-pregnant women, and includes (I) hemolysis or phagocytosis of infected and non-infected erythrocytes, (II) disturbed development of erythrocytes from their precursor cells (dyserythropoiesis) due to hemozoin deposits in the bone marrow and (III) suppression of erythropoiesis as a result of a chronic inflammatory reaction (**Figure 1**) (31). (I) The elimination of non-infected erythrocytes is estimated to be almost 10 times higher than that of infected erythrocytes and thus plays also an important role in the development of anemia (32). Several potential reasons for this phenomenon are under discussion: hypersplenism and the increased activation of macrophages contribute to generally increased phagocytosis and cell lysis (33–36); non-infected erythrocytes also exhibit reduced deformability and are thus degraded in the spleen (37, 38); and the deposition of immune complexes and complement factors on non-infected erythrocytes that may cause receptor-mediated phagocytosis by macrophages (39–41).

(II) The malaria pigment hemozoin directly stimulates the apoptosis of erythrorid progenitor cells (42–44). *In vivo*, elevated plasma hemozoin concentration is associated with anemia and suppression of reticulocytes. The number of pigmented precursor cells in bone marrow correlates with the degree of abnormal erythrocyte development (43). Hemozoin also indirectly impairs erythropoiesis through inflammatory mediators such as TNF and nitric oxide (NO) from activated mononuclear cells (42). In *in vitro* experiments, NO synthesis in monocytes is stimulated by hemozoin. Increased nitric oxide production is associated with decreased hemoglobin levels in children with malaria anemia (45). In contrast, macrophages seem to protect the bone marrow from toxic effects of hemozoin (44).

(III) A further aspect in the development of malaria anemia is the suppression of erythropoiesis due to an inflammatory reaction. Malaria anemia is mainly caused by dysregulation of pro- and anti-inflammatory cytokines, chemokines, growth factors and effector molecules (31). Increased levels of TNF, IL-6 and IL-8 as well as decreased IL-10:TNF or TGF-β1:TNF ratios are associated with anemia (46–51). Proinflammatory cytokines also lead to hypoferremia with reduced hemopoiesis, a major mechanism of inflammatory anemia (52, 53). IL-6 stimulates the synthesis of the iron regulatory peptide hepcidin in the liver, which inhibits intestinal iron resorption and the release of iron from hepatocytes and reticuloendothelial cells (54, 55). In an experimental study on *P. falciparum* infection in five voluntarily participating adults, only slightly elevated levels of IL-6 and hepcidin were measured in serum, but a clear hypoferremia with strongly reduced hemoglobin concentration in the reticulocytes has been reported. The results suggest that the inflammatory disorder of iron hemostasis promotes the development of malaria anemia (56).

Several studies have described a link between maternal anemia and fetal development disorders (27, 29, 57). The reduced number of red blood cells (RBCs) together with altered properties of parasitized RBCs (PRBCs) result in deficient transport of oxygen and CO_2 in the bloodstream. As consequences, chronic hypoxia and elevated oxidative stress arise in the maternal-fetal interface contributing to the occurrence of IUGR (58, 59) (**Figure 1**).

Placental Malaria

Placental malaria is characterized by sequestration of peripheral erythrocytes in the intervillous space and the activation of immune responses leading to inflammation. The manifestation and extent of IUGR have been associated with the severity of placental damage promoted by *P. falciparum* infection. Numerous mechanisms are associated with placental dysfunction and IUGR, including inadequate trophoblast functions, disturbed transport of nutrients, morphological changes, and abnormal angiogenesis. In the following sections, the association between *P. falciparum* infection and these processes will be further discussed in the context of placental malaria. **Figure 1** illustrates the pathogenic mechanisms of maternal anemia and placental malaria that contribute to IUGR.

STRUCTURAL, CELLULAR, AND MOLECULAR MECHANISMS LINKING PLACENTAL MALARIA AND IUGR

Histopathological Changes in the Placenta

Decreased placental weight was detected in women with malaria infection and associated with the presence of placental inflammation and damage (60). In histological samples from patients with active *P. falciparum* infection, erythrocytes in the intervillous space are packed with parasites. In case of past infections, but especially in active chronic infection, the malaria pigment hemozoin can be detected in migrated monocytes or in fibrin deposits (61). Hemozoin deposits without parasitemia indicate an inactive infection (7, 62). Acute infection is more likely to be associated with preterm birth, whilst chronic infection is associated with maternal anemia and reduced birth weight due to IUGR (63). Further histological changes, especially in chronic inflammation, are fibrin deposition, clumping of syncytiotrophoblast cells, reduction of their microvilli, focal necrosis, and thickening of the trophoblast basal membrane (61, 62, 64, 65). These deposits can disturb blood flow in the placenta causing hypoxia and contributing to IUGR (**Figure 1**).

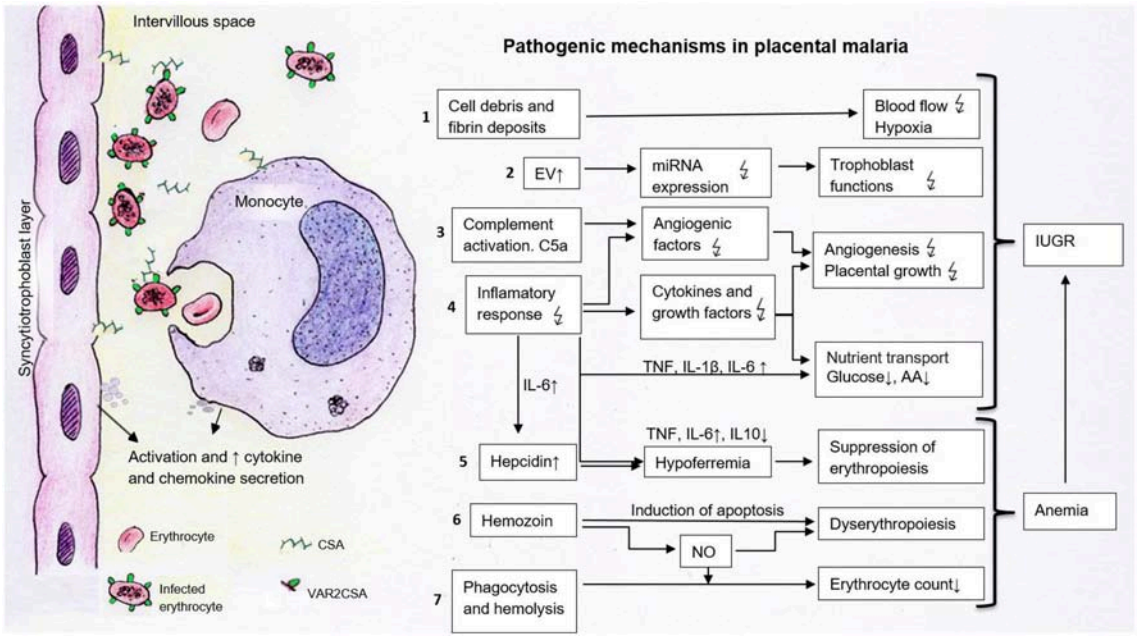

FIGURE 1 | Pathogenetic processes potentially contributing to the development of IUGR in placental malaria. The sequestration of infected erythrocytes expressing VAR2CSA in the intervillous space occurs through the binding to placental chondroitin sulfate (CSA). This process leads to the activation of syncytiotrophoblast cells and local monocytes. These cells secrete chemokines and cytokines, which attract further immune cells which contribute to the pathogenesis of fetal growth restriction. 1. Cell debris and fibrin deposits can disrupt placental blood flow and lead to hypoxia. 2. Elevated EV may carry modified patterns of miRNAs disturbing trophoblast functions. 3–4. Activated complement factors (C5a) and inflammatory responses alter the concentration of cytokines, angiogenic and growth factors, affecting vascularization and placental growth. Cytokines and decreased growth factors impact placental nutrient transporters, resulting in decreased amino acid and glucose transport. 5. The suppressed erythropoiesis is caused either directly via cytokines or indirectly via increased hepatic hepcidin and lowered iron levels. 6. Dyserythropoiesis is a consequence of hemozoin deposits in the bone marrow, which disturb blood formation directly via apoptosis induction or indirectly via inflammatory mediators such as nitric oxide (NO). 7. Phagocytosis and hemolysis reduce erythrocyte numbers contributing to anemia. Together with placental alterations, maternal anemia leads to fetal growth restriction. ↯, disturbed; ↑, increased; ↓, decreased; AA, amino acids; C5a, activated complement factor 5; CSA, chondroitin sulfate A, EV, extracellular vesicles; IL, interleukin; NO, nitric oxide; TNF, tumor necrosis factor.

Altered Inflammatory Response in the Placenta

Infection with *P. falciparum* leads to an inflammatory reaction in the placenta that shifts the balance between Th1 and Th2 immune responses toward the Th1 pathway leading to release of proinflammatory cytokines and migration of immune cells (5, 66). In dual *ex vivo* perfusion experiments on isolated human placental cotyledons *P. falciparum* induces upregulation of transcription factor c-fos gene expression and release of the macrophage migration inhibitory factor (MIF), whereas expression of other chemokines and proinflammatory cytokines has been discussed as unspecific responses to oxidative and hypoxic stress. The observed release of placental chemokines and cytokines has been directed toward the maternal compartment while only trace amounts have been detected in the fetal circuit (67). By sequestration of infected erythrocytes in the intervillous space, syncytiotrophoblast and local maternal immune cells express elevated levels of chemokines [e.g., macrophage-inflammatory protein 1 (MIP1), monocyte chemoattractant protein 1 (MCP1) and interferon-gamma induced protein 10 (IP10)], which cause an increased migration of monocytes (68–71). By means of phagocytosis, cytokine secretion and antigen presentation to T cells, they contribute strongly to the elimination of the pathogens (72). On the other hand, excessive monocyte infiltration contributes to malaria pathogenesis and correlates in numerous studies with negative consequences such as reduced birth weight, premature birth and maternal anemia (73–78). The role of natural killer cells (NK cells) in the placental immune response is controversially discussed. A complete absence of NK cells in the intervillous space of malaria infected placentas has been described, which may be partly responsible for reduced parasite elimination (78). Pregnant women with *P. falciparum* infection have significantly lower levels of INFγ-producing NK cells in the placenta than aparasitemic pregnant women (79). Moreover, the cytotoxicity of NK cells against infected erythrocytes is lower in primiparous women, who are particularly susceptible to malaria, than in multiparous mothers (80). These results suggest that a higher number of INFγ-producing NK cells protects against infection (79, 80). In another study, however, NK cells in infected placentas are elevated and associated with low HLA-G production in trophoblast cells, which may contribute to a negative pregnancy outcome (81).

Monocytes secrete cytokines for the differentiation and activation of further immune cells. The following proinflammatory cytokines can be found elevated in peripheral and/or placental blood of infected pregnant women: TNF

(50, 51, 66, 71, 82–87), IFNγ (50, 51, 66, 71, 82, 83, 85, 88, 89), IL-1/β (66, 84, 86, 89), IL-2 (50, 82) and IL-6 (51, 66). In addition, elevated concentrations of antiinflammatory cytokines such as IL-4 and IL-10 have been detected (51, 66, 71, 82, 84, 85, 90). In some studies IL-2, IL-4, IL-6, IL-10, and TGFβ have been reported as decreased (50, 66, 86). Increased concentrations of TNF are associated with high placental parasite density and pregnancy complications such as LBW, IUGR, preterm birth and maternal anemia (50, 86, 87, 91). In one study, elevated IFNγ has been associated with reduced birth weight (50), while another study did not find this correlation (87) and a further study has shown that reduced IFNγ is associated with reduced birth weight (90). Other authors have found elevated IFNγ in non-infected multiparous women which is associated with the absence of infection and conclude an important role of IFNγ in protecting against infection (85, 89).

Increased IL-10 levels are associated with parasitemia, reduced birth weight and premature birth. The authors assume that IL-10 impedes adequate control of the pathogen (91, 92). In contrast, other authors claim that high IL-10 levels are important to counteract an excessive cellular inflammatory response and to protect against negative pregnancy outcome (50, 66). Since in placental infections IL-10 is also highly elevated in peripheral blood, it has been discussed as a potential biomarker for the diagnosis of infection with *P. falciparum* in pregnancy (84, 90). Furthermore, elevated levels of IL-6 and IL-8 may be associated with high parasite density and anemia, IL-8 with intrauterine growth restriction and IL-7 with absence of infection (51, 86, 89). Lower expression of IL-5 is associated with reduced birth weight (90). **Table 1** summarizes the most important associations of elevated cytokines caused by *P. falciparum* infection with clinical parameters.

Alterations in the levels of placental Th1 and Th2 cytokines and growth factors as a response to *P. falciparum* infection may result in a disbalance which potentially impacts placental functions contributing to IUGR (**Figure 1**).

TABLE 1 | Associations of cytokines with clinical parameters in *P. falciparum* infections in pregnancy.

Elevated cytokine	Association with	References
TNF	High parasite density and monocyte infiltration	(50, 87)
	Low birth weight	(87)
	Premature birth	(91)
	Intrauterine growth restriction	(86)
	Maternal anemia	(50)
	Fetal death in mice	(93)
IFNγ	Low birth weight	(50)
	Fetal death in mice	(93)
	Absence of infection	(85, 89)
	No association with low birth weight	(87)
Low IFNγ	Correlation with low birth weight	(90)
IL-10	Parasitemia and low birth weight	(92)
	Premature birth	(71)
	Control of inflammation	(50, 66)
IL-8	High parasite density and low Hb	(51)
	Intrauterine growth retardation	(86)
IL-7	Absence of infection	(89)
IL-6	High parasite density and low Hb	(51)
Low IL-5	Correlation with low birth weight	(90)

Disruption of Hormonal Balance

Several of the critical hormones in pregnancy are altered in malaria infections, such as the steroid hormones estrogen and progesterone. In addition to their effects on placental development and function, they contribute to the adaptation of the maternal immune system to pregnancy which is crucial for establishing maternal-fetal immunotolerance and prevention of fetal rejection (5, 94). Cortisol, which is secreted by the adrenal glands and associated with stress responses, also performs physiological functions on the placenta and suppresses immune responses (95, 96). The polypeptide hormone prolactin exerts a plethora of effects on maternal metabolism and immune system. It is expressed by the pituitary gland, decidua, myometrium, and immune cells (97).

In infection with *P. falciparum, the* steroid hormones estrogen and progesterone, prolactin and cortisol are altered (89, 98–100). In malaria, estrogen (estradiol, E2) has been found decreased in third trimester peripheral blood (100), and in another study increased in the placenta (89). In the latter study, low levels of estrogen are found especially in multiparous women and are associated with the absence of infection. Pregnancy-maintaining progesterone correlates positively with maternal Hb and child birth weight and is reduced in malaria infection (89). Prolactin has been described as either unchanged or decreased in infected pregnant women (98, 99). Being regulated by progesterone, the dropping of prolactin levels may be related to progesterone shortfall. The concentration of glucocorticoids such as cortisol is significantly increased and associated with high parasite loads (98, 99). Cortisol also correlates with a low number of NK cells and inhibits their cytotoxicity (80, 101).

Changes in the levels of endocrine hormones ocurring in women with malaria may contribute to impaired placental functions and, hence, IUGR. Likewise, hormones have also the potential to interfere with maternal immune responses against the parasite, which potentially influences placental and fetal complications (**Figure 1**). The consequences of the observed hormonal imbalances, however, are poorly understood, and require further investigations.

Disturbed Angiogenesis and Placenta Development

Doppler sonography studies of *P. falciparum* infected pregnant women (32–35 weeks of pregnancy) show abnormal uteroplacental blood flow associated with preterm birth, LBW and perinatal death (102). The underlying pathogenetic processes are complex and not fully understood. Growth and vascularization of the placenta are regulated by various growth factors such as IGF and angiogenesis factors such as angiopoetin (ANG-1/-2), VEGF and its soluble receptor (sVEGFR1) (103).

Altered expression of these factors can severely impair the development of the placenta and the fetus (**Figure 1**). In malaria infected mice, decreased levels of ANG-1, an increased ANG-2/ANG-1 ratio and growth disorders of the fetus have been described (104). Dysregulation of angiopoetin can also be detected in exposed primiparous women and is associated with reduced birth weight (104). Reduced levels of ANG-1 are also associated with various histopathological changes of the placenta in infected pregnant women in areas with low malaria transmission (105). The activation of the complement system, particularly the factor C5, seems to be significantly involved in the pathogenesis of disturbed angiogenesis. In mice, activated C5 (C5a) leads to an increased release of sVEGFR-1 from monocytes, which binds VEGF and makes it ineffective. Important growth stimuli for placental vessels are missing, as a result rejection and fetal growth restriction can occur (106, 107). In case-control studies of pregnant women from Kenya and Malawi, C5a is significantly elevated in placental malaria infection (108, 109). Increased levels of C5a are associated with altered angiogenesis parameters and with babies small for gestational age (109). Levels of IGF-1, an essential growth factor, are significantly reduced in infected pregnant women compared to non-infected ones. Decreased IGF-1 levels also correlate with decreased birth weight (110).

Malaria infection and pre-eclampsia or pregnancy-associated hypertension have some similarities (111). For instance, there is reduced placental perfusion in both pregnancy complications (102, 112). Biomarkers for pre-eclampsia, such as sVEGFR1 and soluble endoglin, are often elevated also in placental *P. falciparum* infection (113–115). An increased risk of hypertension in young primiparous women with chronic malaria infection has been described (114). A similar link between placental infection and pregnancy hypertension has been reported also in a hypoendemic region in Senegal (116). Malaria in pregnancy seems to contribute to the development of pre-eclampsia by placental inflammatory processes with increased cytokine secretion (114–116). Subsequently, preeclampsia constitutes a risk factor for fetal growth restriction (117).

Disorders of Nutrient Transport

Several studies support the hypothesis that in malaria infection, dysregulation of placental nutrient transporters contributes to fetal growth restriction (**Figure 1**). System A transporter, one of the most important amino acid transporters in the placenta, is downregulated in malaria infection (118, 119). In several studies, a reduced function of this transporter has been associated with fetal growth disorders (120–122). Its activity is particularly reduced in placental inflammation with monocyte infiltrate (118). In placental malaria infection, inflammatory mediators inhibit essential signal transduction pathways (119). *In vitro* studies show that proinflammatory cytokines such as 1β, IL-6, and TNF lead to System A transporter dysregulation (123, 124). As described above, they are elevated in malaria infections and associated with reduced birth weight. In addition, growth factors such as IGF-1, which stimulate placental amino acid uptake, are reduced in *P. falciparum infections* (110, 125). In infected placentas, the expression of GLUT-1, a transporter important for basal glucose supply, is also downregulated (126, 127). The GLUT-1 expression at the basal membrane shows a positive correlation with birth weight and a strongly negative correlation with the density of the monocyte infiltrate. These results suggest that the inflammatory response in the intervillous space leads to fetal growth restriction due to impaired transplacental glucose transport (126). In general, increased TNF levels, decreased IGF-1 levels and placental hypoxia are associated with dysregulation of glucose transport (128–130).

Extracellular Vesicles (EV) Containing microRNAs in Malaria

In recent years, extracellular vesicles (EV) have reached the spotlight of intercellular communication, particularly in the maternofetal relationship. Current studies demonstrate that placenta-derived EV are able, for instance, to modulate maternal immune cells (131), platelets (132), and vascular cells (133, 134).

EV are packed with non-coding RNAs, including microRNAs (miRNAs), mRNAs, proteins and lipids, which after internalization, influence the behavior of recipient cells. Several pathological conditions, including preeclampsia (131), IUGR (135), and malaria (136) have been associated with alterations of EV content and functions.

The human placenta possesses a unique profile of miRNA which is dynamically expressed to supply the specific needs of the respective gestational age (137). The study of miRNA expression patterns in placenta tissue has revealed dominant expression of oncogenic, angiogenic, and antiapoptotic miRNAs during the first trimester of pregnancy, whereas the third-trimester is characterized by prevailing expression of miRNAs related to cell differentiation and tumor suppression (138).

Despite the accumulating reports of miRNA expression in IUGR or small for gestational age (SGA) cases associated with preeclampsia (139–141), only few studies have investigated alteration in absence of preeclampsia. Interestingly, these publications suggest an important role of placenta-specific and placenta-associated miRNAs in the development of IUGR. For instance, a report on the placenta-associated miR-141, which is highly enriched in maternal plasma, has shown its overexpression in placentas complicated with IUGR and confirmed the miR-141 target pleiomorphic adenoma gene 1 (PLAG1), which may contribute to the development of this pathology (142). Likewise, seven members of the Chromosome 19 miRNA Cluster (C19MC) are downregulated in placentas from IUGR pregnancies (143).

C19MC is a placenta-specific cluster, low expressed at the beginning of pregnancy, but highly expressed at term (137). Members of C19MC have emerged as important players in the regulation of trophoblast invasion, proliferation and differentiation (144, 145), and more recently, in the immune response to viral infections in pregnancy (146, 147). It has been proposed that C19MC members confer antiviral immunity to trophoblast cells (148) but their role in defense to bacterial or parasite infections remains unknown.

Only a handful of studies have been carried out to investigate the changes in levels of circulating placental EV and miRNAs as

response to malaria infection. Most of these studies have used animal models or had very low number of patients, and thus, have not been included in this review. To our knowledge a single report has been published analyzing expression of these and other placental-miRNAs in plasma EV of pregnant women with malaria (136). The concentration of total and placental-derived microparticles in plasma, characterized by the presence of Pregnancy-Specific Glycoprotein1 (PSG1) on their surface, remains unaltered in women with malaria and increased in those carrying HIV. Independently of malaria or HIV infection, women who delivered growth restricted neonates have higher quantities of both total and placental-derived EV. Furthermore, the level of miRNA-517c, a miRNA belonging to C19MC, is elevated in vesicles isolated from patients with malaria compared to uninfected controls (136). miRNA-517c is also associated with the development of preeclampsia and *in vitro* studies have demonstrated its involvement in decreasing trophoblast invasion and angiogenesis, as well as in increased production sFLT1 contributing to placenta dysfunction (149).

In human subjects, peripheral erythrocytes from malaria infected patients produce higher amounts of EV than from uninfected individuals. The severity of malaria infection and efficacy of anti-malaria therapies can be assessed based on plasma EV content (150, 151). A set of miRNAs is dysregulated during the blood stage of *P. falciparum* infection in adults compared to uninfected subjects. Four miRNAs: miR-1246, miR-6780b-5p, miR-3135b, and miR-6126 were reported as of great importance to malaria pathogenesis due to their involvement in multiple processes, such as cell defense response, immune response, TNF signaling pathway, and T cell receptor signaling pathway (152). A broader analysis of the consensus disease phenocode revealed a group of miRNAs commonly altered in a diverse spectrum of human diseases including autoimmune and infectious diseases. Eighty-eight percent of the miRNAs of the consensus set have the potential to target the principal components of the nuclear import and the inflammasome pathways including KPNA1, NLRP1 (NALP1), and NLRP3 (NALP3) genes. After being upregulated in malaria, these genes return to normal levels in PBMC from patients treated with chloroquine (153), a drug considered in the antimalaria drug policy of the WHO (154) and used for malaria prophylaxis during pregnancy due to its relatively high safety (155).

New miRNA functions have been explored regarding the infection and propagation of malaria. Several studies have demonstrated that *P. falciparum* lack miRNA sequences in its genome, presumably by absence of argonaute and dicer genes (156, 157). However, the presence of approximately 100 human miRNAs was detected within the parasites suggesting a unidirectional transfer (158). During the blood stage of *P. falciparum* malaria infection, uninfected erythrocytes enhance the release of EV which mainly target infected erythrocytes and the parasites therein. An *in vitro* study demonstrated that these EV contain hAgo2-miRNA complexes including those of miR-451 and miR-140, which after internalization by the parasites result in effective downregulation of the essential malaria antigen, PfEMP1 expression (159). This finding supports a report on erythrocytes of sickle cell anemia patients, showing enriched human miR-451 and let-7i in erythrocytes, which are transferred to parasites and target cAMP-dependent protein kinase PKA-R mRNA. This results in inhibition of *P. falciparum* blood stage development and contributes to malaria resistance (158). Monocytes, macrophages, and neutrophils also become activated after being exposed to peripheral erythrocyte EV, indicating their contribution to inflammatory processes taking place during malaria infection (160). These findings highlight the role of miRNAs in the innate resistance of erythrocytes to malaria infection as a host mechanism to minimize disease severity. The study of miRNA in placental infection is also worth to be pursued since the expression of miR-451 and other six placenta-associated miRNAs is altered in primary trophoblast cells exposed to hypoxia and in plasma of pregnant women with IUGR (161).

It is tempting to speculate that EVs produced by *P. falciparum*-infected cells or other cells affected during the infection (e.g., immune cells) as well as their secreted miRNAs may play a role in the pathogenesis of placental malaria and IUGR (**Figure 1**). However, the potential effects and biological mechanisms by which these EV may influence trophoblast cell functions constitute a yet unexplored field. Despite being still at its beginning, the study of EVs and miRNA expression during malaria may contribute to identify novel biomarkers, to understand host immunoregulation and to develop new vaccination and treatments strategies.

INTERACTION BETWEEN VAR2CSA AND CSA IN *P. FALCIPARUM* INFECTION IN THE PLACENTA

The pathogenesis of pregnancy malaria is mainly due to the fact that erythrocytes infected with *P. falciparum* bind to receptors in the placenta and accumulate in the intervillous space (sequestration). According to current research, the interaction between the VAR2CSA protein, a special variant of PfEMP-1 on the surface of infected erythrocytes, and CSA in the intervillous space of the placenta is regarded as the most important binding (162).

Sequestration of infected erythrocytes in the placenta mediated specifically by VAR2CSA has been confirmed using dual side *ex vivo* placenta perfusion (67, 163). Only *P. falciparum* infected erythrocytes expressing VAR2CSA but not those binding to endothelial protein C receptor (EPCR) or lacking PfEMP1 disappear from the maternal circulation and accumulate in the perfused tissue, mostly in the intervillous space and to a less extend on the syncytiotrophoblast (163). Therefore, understanding the molecular and structural processes of VAR2CSA-CSA binding and the mechanisms to inhibit this interaction may offer new intervention options, in particular for vaccine development.

Chondroitin Sulfate a (CSA): Function, Occurrence, and Structure

CSA is a glycosaminoglycan, a sugar chain made up of disaccharide units which, bound to a protein, forms a proteoglycan (164). In 1996, CSA was described as a

target structure for the adhesion of *P. falciparum* infected erythrocytes in the placenta (165). It has been detected by immunohistochemistry in the intervillous space and to a lesser extent at the syncytiotrophoblastic layer. It has been discussed that CSA is produced by the fetus and secreted into the intervillous space (166). In placenta infected with *P. falciparum* significantly higher concentrations of CSA have been found (166, 167). Since it is assumed that proteoglycans are involved in the mobilization of cytokines, hormones and growth factors in tissues, an increased expression of CSA may negatively influence placental functions and promote the development of complications through increased adhesion of parasites (167–169).

The understanding of the exact molecular and structural composition of CSA is fundamental to analyze its interaction with VAR2CSA (164). In addition, it is important for the development of novel therapeutic approaches, such as chondroitin sulfate oligomers that bind to VAR2CSA with a higher affinity than CSA and thus impede the sequestration of infected erythrocytes in the placenta (170).

The *var2csa* Gene in *P. falciparum* Infection

In the presence of a placenta, a *P. falciparum* subpopulation switches to the expression of the *var2csa* gene, one of 60 var genes of PfEMP-1 (171). In contrast to the other var genes, which differ greatly between the strains, *var2csa* is widely conserved (172–175). The *var2csa* gene is detected in the analysis of the genome of *P. falciparum* isolates from different regions in almost all strains (173, 175, 176). Of the 60 var genes, only three, including *var2csa*, seem to be expressed in all three examined *P. falciparum* strains (3D7, IT4, and HB3 strains).

In earlier studies, *var2csa* and other var genes, such as *var1csa* and *varcs2*, have been associated with the adhesion of infected erythrocytes in addition to *var2csa*, but this could not be confirmed in more recent studies (171, 177–180). However, studies investigating the genome and protein synthesis of placental parasites have revealed other highly regulated genes and proteins that might be indirectly involved in the pathogenesis of placental malaria (181–185). While the disruption of the *var2csa* gene leads to the loss of CSA binding capacity (179, 186), no other highly regulated var genes are directly related to placental adhesion (187).

VAR2CSA Protein in *P. falciparum* Infection

VAR2CSA is selectively expressed on the surface of *P. falciparum* infected erythrocytes in the placenta. It was discovered in 2003 by Salanti et al. and confirmed in numerous further studies as the most important ligand of CSA (175, 179, 180, 186–189). VAR2CSA is a large (350 kDa) transmembrane polypeptide. The extracellular part consists of six Duffy binding-like domains (three DBL domains each of class x and ε, omitted in the further text for reasons of clarity), a cysteine-rich interdomain (ID2a/b) and further short interdomain segments (ID1, ID4; **Figure 2** and **Table 2**) (198). VAR2CSA is structurally and functionally very different from other PfEMP-1 proteins. For example, specific domains necessary for the recognition of vascular receptors such as CD36 and ICAM-1 are missing (162, 175). Rosetting—an otherwise important pathogenesis factor that describes accumulation of infected erythrocytes—is also atypical in placental malaria infections (199–201).

The VAR2CSA Duffy Binding-Like Domains

Due to the size and complexity of VAR2CSA, only the quaternary structure of individual domains, but not that of the entire protein, has been described so far (202, 203). The best-known structure is that of the DBL3x domain (DBL3), which binds CSA *in vitro* and has been described in detail in two crystallographic studies (204, 205). DBL3 consists of an α helix with numerous inserted loops and can be divided into three subdomains. A loop between the second and third subdomain, which is disordered in the unbound state, assumes an organized structure in the presence of sulfate or disaccharides and forms a sulfate binding pocket (204). The conformational change creates a positively charged region that attracts the negatively charged CSA. It has been shown that mutations in these areas strongly affect the binding of CSA to DBL3 (205, 206). The flexible loop and other surrounding structures are located on the domain surface, are polymorphic and may protect the CSA binding site from recognition by the immune system (204).

VAR2CSA has numerous polymorphic areas compared to non-var proteins, leading to a high antigenic diversity and different placental *P. falciparum* strains (176, 207). Polymorphisms are important mechanisms for immune evasion and arise under selection pressure through exposure to the host immune system (176, 190, 192). They are mostly located on the protein surface, protect conserved areas from immune defense and hinder the formation of cross-strain antibodies (208–210). By analyzing the amino acid sequence of VAR2CSA and by comparing VAR2CSA sequences of different *P. falciparum* strains, conserved and polymorphic areas can be identified and the DBL domains of VAR2CSA can be further characterized. For instance, the DBL3 domain consists of four highly conserved sequences (C1-4) and three variable sequences (V1-3). Some of the conserved areas are also located on the protein surface and are target structures of naturally acquired antibodies (196, 197) (**Table 2**).

The sequence of the DBL2 region of VAR2CSA shows a so-called "dimorphic" structural motif of 26 amino acid length, which divides the strains into two phylogenetic groups (the FCR3 and the 3D7 strains) (207). Another dimorphic region of 167 amino acid length has been detected in the interdomain 1 (ID1), with 76% of placental isolates from Benin containing the first variant (cluster 1) and 24% the second variant (cluster 2). *P. falciparum* isolates with cluster 2 are associated with both multiple pregnancy and high parasitemia (192). The dimorphic areas seem to assume an essential function in pathogenesis, as they contain important elements for CSA binding and have remained stable for a long time in evolutionary history (192, 207).

The analysis of nearly full-length *var2csa* sequences from parasite isolates form around the world and also those reported at the GenBank and the *P. falciparum* genome sequencing projects (in total 106 *var2csa* sequences) revealed that the six DBL domains differ in amino acid conservation between 61 and 88%

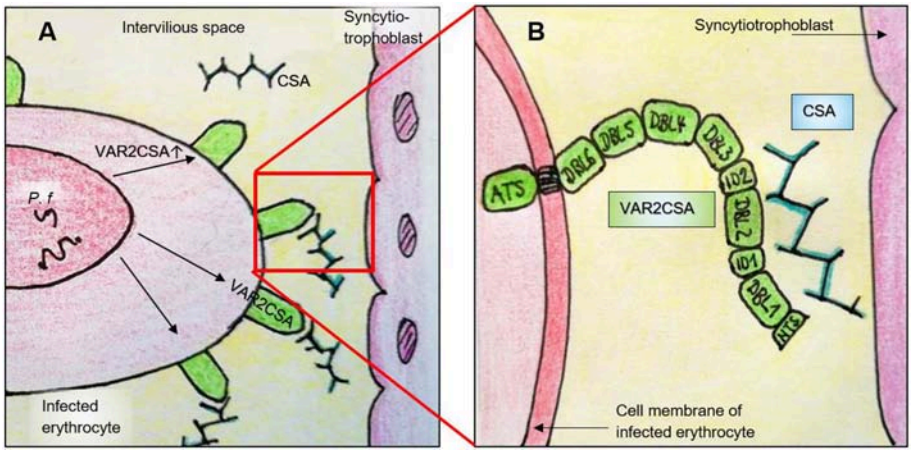

FIGURE 2 | Simplified illustration of the interaction between VAR2CSA and CSA in the placenta. **(A)** *P. falciparum* expresses VAR2CSA on the surface of infected erythrocytes, which in turn binds to CSA in the intervillous space. Pf, *P. falciparum* within a parasitophorous vacuole; CSA, chondroitin sulfate A. **(B)** Enlarged view of **(A)**. VAR2CSA binds to CSA with its N-terminal domains. ATS, Acidic terminal segment; DBL, Duffy-binding-like domain; ID, Interdomain; NTS, N-terminal segment.

TABLE 2 | Structure and sequence polymorphism of VAR2CSA domains relevant for CSA interaction and sequestration of *P. falciparum* in the placenta.

Domain	Structure and sequence polymorphism	Strains in which domain binds to CSA	Strains in which multidomains bind to CSA		
DBL1	7 VBs, 4 SCBs (190)	Does not bind to CSA			
Interdomain 1 (ID1)	Dimorphic region of 167 amino acid length; 78% variant (cluster 1) and 24% the second variant (cluster 2) (192)	Does not bind to CSA	3D7 (191)		
DBL2	8 VBs, 4 SCBs (190) Dimorphic structural motif of 26 amino acid length, which classifies two phylogenetic groups (the FCR3 and the 3D7 strain) (194)	3D7, FCR3		FCR3 (193)	FCR3 (195)
Interdomain 2 (ID2)	Not described	Not described			
DBL3	5 VBs, 4 SCBs (190) 3 variable sequences (V1-3) and four highly conserved sequences (C1-4) (196, 197)	3D7, FCR3			
DBL4	5 VBs, 4 SCBs (190)	Does not bind to CSA			
DBL5	7 VBs, 4 SCBs (190)	3D7			
DBL6	6 VBs, 4 SCBs (190)	3D7			

VB, variable block; SCB, semi-conserved blocks.

and are also structured into variable blocks (VB) and semi-conserved blocks (SCB, block B, D, F, and H) (190). DBL6 is the least conserved VAR2CSA domain with seven variable blocks consisting of a limited number of consensus sequences, i.e., similar or identical sequence patterns (211). Within DBL-6, the variable blocks 1 and 5 (VB1, VB5) from different parasite strains are recognized cross-reactively by antibodies from the plasma of exposed pregnant women (202, 212) (**Table 2**).

The gene diversity of *var2csa* is based on a high rate of self-recombination with a limited repertoire of sequences. In many variable regions the polymorphism is limited by already known or similar structural motifs (176, 190, 213, 214). As they may cause cross-reactive antibody production, these globally shared structural motifs as well as the conserved surface-exposed areas of VAR2CSA are of particular interest for the vaccine development (190, 196, 213). A summary of the structure and gene polymorphisms of individual DBL motifs are presented in **Table 2**.

Interaction of VAR2CSA and CSA

To test if individual domains of VAR2CSA can independently bind to CSA, the DBL domains of two *P. falciparum* laboratory strains, the 3D7 and FCR3 strains, have been produced recombinantly and their binding to immobilized CSA has been measured *in vitro*. Four domains (DBL2, DBL3, DBL5, and DBL6) which bind to CSA have been found (**Table 2**). However, the results vary depending on the study and strain: from the 3D7 strain DBL2 (188, 215–218), DBL3 (196, 206, 216, 218), DBL5 (215, 218), and DBL6 (188, 206, 215, 217) and from the FCR3 strain, only DBL2 (188, 217) and DBL3 (188, 196, 205, 217) bind to CSA (**Table 2**).

Binding specificity and affinity of individual DBL domains also differ greatly from that of the entire extracellular section of VAR2CSA. Although some authors have demonstrated CSA-specific binding (188, 205, 216), the addition of CSC (chondroitin sulfate C) or HA (hyaluron sulfate) affects the binding to CSA of individual DBL domains but not of the entire VAR2CSA protein (217). Furthermore, DBL3 and DBL6 domains of the 3D7 strain bind nonspecifically various glycosaminoglycans, especially those with high sulfonation and many negative charges (206). Compared to the entire extracellular part of VAR2CSA, the affinity of the individual domains to CSA is up to 100,000 times lower. While concentrations of the entire protein in the nanomolar range are sufficient to bind >50% of CSA, micromolar concentrations are necessary for individual domains (191, 219, 220). These results suggest that individual domains do not have the same functional capacity as the entire VAR2CSA protein and that a specific and highly affine CSA binding requires multiple domains. This has been supported by studies demonstrating that combined domains in the N-terminal region of VAR2CSA can bind CSA with similar affinity as the whole protein (191, 196). According to Clausen et al., the minimal CSA binding region is located in the small ID1-DBL2b range, with DBL2b reaching up to 93 amino acids into the ID2a segment. Since ID1-DBL2 does not bind to CSA and ID1-DBL2b binds with high affinity, these 93 amino acids of ID2a appear to play an important role in the interaction with CSA. Although the ID1 region does not seem to be essential for direct binding, it is essential for the formation of a functional CSA binding protein (193). In two other studies, the core region of binding was found in the multidomains DBL1-DBL2/DBL3 (191) and DBL2-ID2b (195). Except for DBL2-ID2b and DBL1-ID2b all VAR2CSA fragments show specificity for CSA (193, 195). In summary, by combining several N-terminal domains around DBL2, a high CSA specificity and affinity can be achieved (**Figure 2** and **Table 2**).

The spatial structure of VAR2CSA is very complex. In order to establish highly specific and affine binding to low sulfonated CSA, several domains of VAR2CSA appear to come into contact with each other and form a quaternary structure (210, 219, 220). This creates specific pockets, loops and structures that interact with the various functional groups of CSA (164). It can be assumed that VAR2CSA is an allosteric protein with positive cooperativity. This means that the binding of a functional CSA group to a domain of VAR2CSA changes the conformation of the protein in a way that the binding affinity for further CSA groups increases progressively (198).

Further Relevant Binding Structures

It is under discussion whether other receptors besides CSA, including hyaluron sulfate (HA) and the Fc part of non-specific antibodies, contribute to the pathogenesis of pregnancy malaria (221, 222). Placental isolates can also bind to HA *in vitro* and at least some *P. falciparum* strains recognize both CSA and HA (221, 223). Placental isolates from Uganda show binding to CSA, HA and non-specific IgG and IgM (224). Further studies confirm the binding of the Fc part of IgM to VAR2CSA, which supports the hypothesis of immune evasion via unspecific blocking (200, 225–227). Other studies did not find significant binding of placenta isolates to HA or IgG antibodies (187, 219, 220, 228). Furthermore, HA does not appear in intervillous space, indicating that an essential role in pathogenesis is unlikely (167). As CSA is currently considered the main receptor for placental parasites, vaccine development focusses on inhibiting CSA-VAR2CSA interaction (193, 228). However, additional interactions, such as the immune evasion of parasites by shielding with IgM, can strongly influence the success of a vaccine or may lead to development of novel targets (163, 225).

VAR2CSA for Vaccine Development

VAR2CSA is the leading candidate for a vaccine against malaria in pregnancy. Besides other preventive strategies the vaccine may be given to girls before puberty. This contact with the VAR2CSA protein leads to immunity against VAR2CSA-expressing *P. falciparum* which, in pregnancy, prevents the sequestration in the placenta (229).

Polymorphism and size (350 kDA) of VAR2CSA protein remain two major challenges that, thus far, have prevented the production of the entire protein for vaccination purposes (230). Therefore, the focus of current research is the identification of the minimal binding area within VAR2CSA, which has a high CSA binding affinity and specificity similar to that of the entire protein, and which simultaneously, induces broad binding-inhibitory antibody production (213).

As described before, this minimal binding region is located in the N-terminal region of VAR2CSA (191, 193, 195). Further studies have examined naturally acquired antibodies in multigravida in endemic regions (231–234) or the expression of antibodies against the VAR2CSA-CSA complex in laboratory animals and *in vitro* (195, 235–239). Two multidomains (ID1-ID2a, DBL1-DBL2) of VAR2CSA have been found as promising vaccine candidates (191, 193, 236, 238–240). They are part of two placental malaria vaccine projects, the PlacMalVac and PRIMALVAC project, which started phase I clinical trials in 2013 and 2016, respectively (241, 242). According to current information, PlacMalVac is safe, well-tolerated and a phase II clinical trial is under preparation (242, 243).

CONCLUSIONS

P. falciparum infection in pregnancy leads to a specific involvement of the placenta where infected erythrocytes express the unique antigen VAR2CSA which binds to CSA in the intervillous space leading to their sequestration. This process induces an inflammatory reaction of the placenta that activates monocytes to switch the release pattern of soluble factors. The consequences are manifold and include disorders of erythropoiesis, angiogenesis, blood flow, and nutrient transport which together impact placental growth, and finally, fetal growth.

The knowledge on the specific VAR2CSA expression and its detailed structure and binding to placental CSA has led to the development of novel vaccine strategies which have a high potential to reduce *P. falciparum* induced pregnancy disorders and IUGR in the near future.

AUTHOR CONTRIBUTIONS

UM has coordinated the writing and has done revisions and corrections on the manuscript. JS has written most parts of the manuscript. RF and DM-P have added the chapter on EV and miRNA and have done several modifications of the text. HS has critically read and corrected the manuscript.

FUNDING

This work was supported by research grants from the German Research Society (DFG, grant Mo2017/2 and Mo2017/3 to DM-P and Ma1550/12-1 to UM and RF).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 11 March 2019
doi: 10.3389/fendo.2019.00124

Influence of Low Protein Diet-Induced Fetal Growth Restriction on the Neuroplacental Corticosterone Axis in the Rat

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *16 September 2018*
Accepted: *11 February 2019*
Published: *11 March 2019*

Citation:
Schmidt M, Rauh M, Schmid MC, Huebner H, Ruebner M, Wachtveitl R, Cordasic N, Rascher W, Menendez-Castro C, Hartner A and Fahlbusch FB (2019) Influence of Low Protein Diet-Induced Fetal Growth Restriction on the Neuroplacental Corticosterone Axis in the Rat. Front. Endocrinol. 10:124. doi: 10.3389/fendo.2019.00124

Objectives: Placental steroid metabolism is linked to the fetal hypothalamus-pituitary-adrenal axis. Intrauterine growth restriction (IUGR) might alter this cross-talk and lead to maternal stress, in turn contributing to the pathogenesis of anxiety-related disorders of the offspring, which might be mediated by fetal overexposure to, or a reduced local enzymatic protection against maternal glucocorticoids. So far, direct evidence of altered levels of circulating/local glucocorticoids is scarce. Liquid chromatography tandem-mass spectrometry (LC-MS/MS) allows quantitative endocrine assessment of blood and tissue. Using a rat model of maternal protein restriction (low protein [LP] vs. normal protein [NP]) to induce IUGR, we analyzed fetal and maternal steroid levels via LC-MS/MS along with the local expression of 11beta-hydroxysteroid-dehydrogenase (*Hsd11b*).

Methods: Pregnant Wistar dams were fed a low protein (8%, LP; IUGR) or an isocaloric normal protein diet (17%, NP; controls). At E18.5, the expression of *Hsd11b1* and *2* was determined by RT-PCR in fetal placenta and brain. Steroid profiling of maternal and fetal whole blood, fetal brain, and placenta was performed via LC-MS/MS.

Results: In animals with LP-induced reduced body ($p < 0.001$) and placental weights ($p < 0.05$) we did not observe any difference in the expressional *Hsd11b1/2*-ratio in brain or placenta. Moreover, LP diet did not alter corticosterone (Cort) or 11-dehydrocorticosterone (DH-Cort) levels in dams, while fetal whole blood levels of Cort were significantly lower in the LP group ($p < 0.001$) and concomitantly in LP brain ($p = 0.003$) and LP placenta ($p = 0.002$). Maternal and fetal progesterone levels (whole blood and tissue) were not influenced by LP diet.

Conclusion: Various rat models of intrauterine stress show profound alterations in placental Hsd11b2 gatekeeper function and fetal overexposure to corticosterone. In contrast, LP diet in our model induced IUGR without altering maternal steroid levels or placental enzymatic glucocorticoid barrier function. In fact, IUGR offspring showed significantly reduced levels of circulating and local corticosterone. Thus, our LP model

might not represent a genuine model of intrauterine stress. Hypothetically, the observed changes might reflect a fetal attempt to maintain anabolic conditions in the light of protein restriction to sustain regular brain development. This may contribute to fetal origins of later neurodevelopmental sequelae.

Keywords: IUGR intrauterine growth restriction, steroids, brain, placenta, Hsd11b2, rat model, low protein, neuroplacentology

INTRODUCTION

The association of intrauterine growth restriction (IUGR) with the development of metabolic disorders [i.e., type 2 diabetes, hyperlipidemia (1)] and cardiac disease [i.e., arterial hypertension, cardiovascular disease (2)] in later life is well-recognized based on several animal (3, 4) and human (5) studies. Barker introduced the hypothesis of "fetal programming," postulating that an adverse intrauterine environment affects growth and differentiation of the fetus and subsequently contributes to the development of the above sequelae later in life (6).

The mechanistic basis of "developmental origins of health and disease" (DOHaD) are subject of ongoing research (7). A possible mechanism that links maternal gestational adversity to long-term health outcomes in offspring is the diaplacental transfer of glucocorticoids (8): during pregnancy the placental enzyme 11beta-hydroxysteroid dehydrogenase 2 (Hsd11b2) catalyses the oxidation of cortisol to cortisone [predominant in humans (9)] and of corticosterone (Cort) to 11-dehydrocorticosterone [DH-Cort, predominant in rats (9)], thereby inactivating these glucocorticoids. There is evidence that low birth weight might be associated with a reduced placental HSD11b2 activity in both humans (10) and rodents (11–13) contributing to fetal hypercortisolism and programming of hypertension and metabolic syndrome in adult rat offspring.

Beyond these major schemes of fetal programming, a potential negative influence of poor fetal growth on brain development with anxiety-related sequelae is being critically discussed (14). In human pregnancy, heightened maternal anxiety with endogenous elevation of glucocorticoids might predispose the offspring for schizophrenia (15), hyperactive disorder (16) and/or impaired emotional and cognitive development (17). It has been shown that maternal anxiety is negatively correlated with placental *HSD11b2* mRNA expression (18). Additionally, a reduction of placental HSD11b2 seems to be associated with IUGR (19). We have also found that the *HSD11b2* mRNA expression is negatively correlated with postnatal catch-up growth in IUGR (10).

There is evidence that iatrogenic administration of exogenous glucocorticoids for lung maturation might exert negative fetal effects: A single course of antenatal betamethasone (not metabolized by HSD11b2) treatment before 34 + 0 weeks of gestation seems to be associated with an impairment of cognitive ability in treated infants (20, 21) and a reduced head circumference in females (22) at term. Moreover, betamethasone treatment (23) and inhibition of HSD11b2 activity (24) was associated with a certain impairment of hypothalamic-pituitary-adrenal (HPA)-axis function in humans.

Similar to humans, fetal exposure of rats to gestational stress (e.g., via inhibition of Hsd11b2 or treatment with glucocorticoids) seemed to induce low birth weight and trigger processes in the HPA-axis with negative effects on postnatal neurodevelopment. Affected rats showed increased stress responsivity, anxiety-like behavior and altered social interaction postnatally (25–28). Experiments with Hsd11b2 null mice are indicative of direct fetal programming effects on animal behavior via endogenous glucocorticoids (29).

Various non-surgical rat models for the induction of gestational maternal adversity exist (28). In general, maternal stress is either directly [e.g., chronic restraint stress (30)] or indirectly [e.g., nutritional restriction (13, 31)], induced during various stages of gestation. Maternal glucocorticoids are deemed to be among the main effectors of fetal programming in these animals (32), as prenatal effects can be averted via adrenalectomy of the dams (33). Noteworthy, the glucocorticoid system is known to be tightly interlinked with other essential regulators of fetal neurodevelopment, such as the neuroplacental serotonin (5-hydroxytryptamine; 5-HT) axis (34, 35). Perturbations of the fetal serotonin system during early development by prenatal maternal stress have been linked to processes of fetal programming of psychiatric disorders in later life (35).

In our study we investigated the maternal and fetal (E18.5) HPA-axis in an established nutritionally-induced IUGR model (36), using a novel methodological approach. So far, endocrine changes in such models were mainly studied using multiple ELISA measurements of single hormones in serum or RT-PCR of *Hsd11b2* in target tissues (i.e., placenta and brain) (11, 12, 22, 37–40). We have recently established a liquid chromatography tandem mass-spectrometry method (LC-MS/MS) that allows for the detection of multiple glucocorticoids in a single tissue/serum probe (9, 41), minimizing tissue-matrix interactions (42, 43). The combination of LC-MS/MS with volumetric adhesive microsampling devices enables individual sampling without the need for pooling of fetal samples (44). We set out to determine the steroid profile in maternal and fetal circulation, along with placental and brain steroid profiles, in IUGR vs. control rats and to compare these findings to *Hsd11b2* mRNA expression in these tissues. We were especially interested to identify steroid profiles characteristic for IUGR. In the face of potential IUGR-related neurologic sequelae and the role of placental Hsd11b2, we aimed to provide evidence of a neuroplacental cross-talk on the glucocorticoid level in these animals.

METHODS

Animals and Diets

Animal procedures were carried out as previously described (36). This study was carried out in accordance with the recommendations of the NIH *Guide for the Care and Use of Laboratory Animals* and the *EU Directive 2010/63/EU*. All procedures and protocols were governmentally approved by the corresponding board (Regierung von Mittelfranken, AZ #54-2531.31-31/09). Wistar rats were ordered from Charles River (Sulzfeld, Germany). Virgin female rats (240–260 g) were housed individually and maintained at 22°C on a 12 h light–dark cycle. After mating, pregnancy was confirmed via vaginal plug formation (day 1 of gestation). The use of our alimentary IUGR model for the analysis of postnatal sequelae of fetal programming has been previously described by us (3, 36, 45, 46). In short, 12 dams were randomly assigned to two groups receiving semi-purified diets (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) of either low protein diet (LP group, 25 g/d of Altromin C1003, 8.1% protein, 13% fat, 78% carbohydrates [2% monosaccharides, 18% disaccharides, 49% polysaccharides] or an isocaloric diet of normal protein content (NP group, 25 g/d of Altromin C1000, 17.3% protein, 13% fat, 67% carbohydrates [10% disaccharides, 47% polysaccharides], and were weighed daily. Animal characteristics are displayed in **Table 1**.

Additionally, a second set of four dams (conversion set) was mated and received NP diet *ad libitum*. Blood samples of these animals and the respective fetuses were used to establish a conversion factor for volumetric adhesive microsampling (VAMS) to EDTA-blood in order to improve comparability of data with findings from the literature (**Supplementary Table S1**). Moreover, LC-MS/MS pretesting of placental tissue and brain in these animals helped to optimize run settings for steroid and CRH detection.

Sample and Data Collection

At E18.5 fetuses were obtained via cesarean section under isoflurane anesthesia. Starting out with six dams per group, one dam of the NP group (NP) had to be excluded as litter size (<10 fetuses) was extremely low. Additionally, two dams (one NP and LP, each) did not follow their expected growth trajectories and were subsequently excluded by the veterinarian due to suspected health issues. For our final analysis six fetuses ($n = 3$ females, $n = 3$ males each) per dam ($n = 4$ NP, $n = 5$ LP) were chosen based on their matching positions in the proximal uterine horns. Fetuses were sacrificed via decapitation, followed by individual Mitra™ (Neoteryx, LLC, Torrance, CA, USA) VAMS of mixed arterio-venous blood from the trunk's cutting surface, as described (44). Brain and placenta samples were instantly snap-frozen in liquid nitrogen and stored at −80°C. Fetal tail-tip biopsies were used for sex determination. We did not perform fetal saline-perfusion prior to tissue collection, as this procedure has been shown to interfere with cerebral tissue steroid detection (47). Subsequently, dams were sacrificed via aortic transection. Arterial blood was drawn via syringe and collected in K3 EDTA blood collection tubes (Sarstedt, Nümbrecht, Germany). Additionally, whole blood samples were collected via VAMS.

In the conversion set of animals (see above), trunk blood of fetuses from one litter was pooled in EDTA-tubes to facilitate the measurement of a maximum number of steroids for the purpose of converting VAMS steroid measurements into EDTA standard values (**Supplementary Table S1**).

TABLE 1 | Rat auxologic data.

Weight (g)	NP	LP	*p*-value
Litter (*n*)	4	5	
Fetus	1.38 ± 0.09	0.84 ± 0.06	<0.001[#]
Brain	0.08 ± 0.01	0.08 ± 0.05	ns[§]
Placenta	0.34 ± 0.04	0.29 ± 0.02	<0.050[#]
Placenta/Fetus	0.25 ± 0.02	0.35 ± 0.03	<0.001[#]
Litter size	12.75 ± 2.22	14.20 ± 2.59	ns[#]

(#) Welch's t-test, (§) Mann–Whitney U-test.

RNA Extraction, RT-PCR, and Real-Time Quantitative PCR

For measurement of *Hsd11b1* and *2*, RNA was extracted from fetal placenta and brain using peqGOLD TriFast™, according to the manufacturer's instruction (VWR International GmbH, Darmstadt, Germany). RNA concentration was quantified via NanoDrop ND1000 spectrophotometry (Peqlab) and adjusted to 1 ng/ml. After DNase treatment, RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) and random hexamers as primers. Reactions without Quantiscript Reverse Transcriptase served as negative controls. Quantitative real-time PCR was performed with SYBR Green (Applied Biosystems, Darmstadt, Germany; ThermoFisher Scientific) using the StepOnePlus Real-Time PCR System (Applied Biosystems). Samples were run in duplicates and mRNA levels were normalized to the housekeeping gene *18S* rRNA. SYBR-Green based real-time PCR results were compared between groups using the $2^{-\Delta\Delta CT}$-method. Primers sequences were as following (5′-3′): *r18s* forward (fw) TTGATTAAGTCC CTGCCCTTTGT, *r18s* reverse (rev) CGATCCGAGGGCCTC ACTA; *Hsd11b1* fw TAGACACAGAAACAGCTTTG, *Hsd11b1* rev AATTCCATGATCCTCCTTCC; *Hsd11b2* fw CAGGAGACA TGCCATACC, *Hsd11b2* rev GATGATGCTGACCTTGATAC.

Sex verification was carried out via sex-determining region Y (*Sry*) gene PCR (45). DNA from tail-tip biopsies was extracted using the MyTaq™ Extract-PCR Kit (Bioline, London, UK). Sex determination was performed via commercially available PCR *Sry* assay (Rn04224592; ThermoScientific, Waltham, USA) with *18S* rRNA as housekeeping gene. PCR conditions were adopted from the manufacturer's protocol (ThermoFisher). For male gender a cut-off <23 cycles was chosen, while the threshold for female sex was >29 cycles. When verification was needed, we repeated the measurement with corresponding liver samples.

Liquid Chromatography—Tandem Mass Spectrometry (LC-MS/MS)

We have previously established and validated LC-MS/MS methods for the determination of steroids in human placenta (41)

and amniotic fluid (48), as well as in rat placenta and brain (9). We determined corticosterone (Cort), 11-dehydrocorticosterone (DH-Cort), 11-deoxycorticosterone (DOC), progesterone, androstenedione, and testosterone in maternal and fetal whole blood, fetal brain, and placenta. While others were able to detect testosterone in pooled fetal plasma on E19 by RIA (49), androstenedione and testosterone were below the detection limit in whole blood (data not shown), due to individual sampling. LC-MS/MS allows for the combined quantification of corticotropin-releasing hormone (CRH) and steroids. As CRH is not present in rat placenta (9), our study involves its determination in fetal brain only. The following adjustments were made to the methods above: We used 1 ml of ethanol solvent containing protease inhibitor cocktail (Aprotinin 2.0 µl/ml, Pepstatin 6.7 µl/ml and Leupeptin 10.0 µl/ml, all from Carl Roth, Karlsruhe, Germany) per 150 mg tissue for homogenization and waived ultrasonification based on tissue softness. The reduced fetal organ size allowed for the reduction of the amount of resuspended tissue homogenate to 1/10th. Thus, 30 µl of homogenized supernatant were added to 90 µl methanol (MeOH) containing 1% formic acid (FA) for LC-MS/MS sample preparation. Thereby, we could maintain our established sample to solution ratio of 1:3. For steroid profiling, sample protein precipitation was adjusted to the initial reduction of homogenate quantity, such that 50 µl of the above methanol/FA supernatant was added to 50 µl MeOH/zinc sulfate (50 g/L, 1/1 v/v), while the addition of 100 µl internal standard remained unmodified (41). The concentration of standards dilution series encompassed a range of 1.0−250.0 ng/ml. CRH quantification was performed with the remaining homogenate *in toto*. We added MeOH/FA (99/1 v/v) containing 0.24 µg/ml bovine CRH as internal standard (IS, Bachem AG, Bubendorf, Switzerland) relative to 1/3rd of the homogenate volume. Serial standard dilutions of CRH with equivalent IS-ratio covered a detection range of 0.1–20.0 ng/ml. LC-MS/MS measurements of steroids in VAMS of fetal whole blood via MITRA capillary blood collection device (Neoteryx LLC, Torrance, CA, USA) has been previously established and validated by us (44). The final fetal and maternal conversion factors from VAMS whole blood to EDTA blood are given in **Supplementary Table S1**.

Statistical Analysis

Data processing and imaging was performed with Microsoft Office 2016 (Microsoft, Redmond, WA, USA) and Adobe Photoshop CS6 (Adobe Systems, San José, CA, USA). For statistical analysis we used GraphPad PRISM Version 7.0 (GraphPad Software, La Jolla, CA, USA). For every litter, the mean levels of the respective individual fetal hormones in tissue and blood were calculated. Outliers of all laboratory parameters and respective ratios within litters and groups were corrected by using the PRISM "robust regression and outlier removal" (ROUT) method (Q = 1%, equivalent to a false discovery rate of 1%), as described by Motulsky and Brown (50) and Hughes and Hekimi (51). Excluded data points ($n = 6$) are given in **Supplementary Table S2** and were not included in the calculation of the mean per litter. Subsequently, the means per litter ($n = 4$ for the NP group, $n = 5$ for the LP group) were subjected to further statistical analysis. Before performing groupwise comparisons, we checked the normality assumption of the mean values by carrying out a Shapiro Wilk test in each experimental group, followed by a Bonferroni correction of the resulting p-values (one per experimental group). The null hypothesis of normality in both groups was rejected if at least one Bonferroni-corrected Shapiro Wilk $p < 0.05$. When normal distribution was present, Welch's t-test was performed, otherwise a Mann–Whitney U-test was executed. For sex-specific subgroup comparison between NP and LP groups, we used two-way analysis of variance (two-way ANOVA with Sidak's multiple comparison analysis). The normality assumption for the ANOVA residuals was verified using normal quantile-quantile (QQ) plots. All data in this manuscript represent mean ± standard deviation (SD) regardless of their normality, facilitating comparative overview. A $p < 0.05$ was considered statistically significant.

RESULTS

Auxologic Data

We did not observe significant differences in litter size in our LP group secondary to their dietary restriction (**Table 1**). The fetal and placental weights were both significantly lower in the LP group compared to the NP group ($p < 0.001$ and $p < 0.05$, respectively, **Table 1**), while brain weight did not differ (**Table 1**). The ratio of placenta-to-body weight was significantly increased in the LP group ($p < 0.001$, **Table 1**). Thus, while LP diet negatively affected both body and placental weight, its influence on fetal body weight appeared to be more prominent (**Table 1**).

Quantitative Real-Time PCR (qRT-PCR)

The results of our qRT-PCR of fetal brain and placental tissue are listed in **Table 2**. We did not detect significant differences in *Hsd11b1* and *2* mRNA expressions between the NP and LP groups in both brain and placenta. Moreover, the ratio of *Hsd11b1* to *Hsd11b2* was not significantly influenced by LP diet in brain (**Figure 1A**) and placenta (**Figure 1B**). Sex-specific analysis of *Hsd11b1/2*-ratio between NP and LP groups did not reveal significant differences (data not shown).

Steroid Analysis via LC-MS/MS in Maternal and Fetal Whole Blood

Steroid profiles of maternal, as well as of fetal whole blood can be found in **Tables 3**, **4**, respectively. NP and LP dams showed

TABLE 2 | qRT-PCR results.

		NP	LP	*p*-value
Brain	*n*	4	5	
	Fold change (*Hsd11b1*)	1.00 ± 0.69	0.89 ± 0.94	ns[#]
	Fold change (*Hsd11b2*)	1.00 ± 0.51	1.11 ± 0.53	ns[#]
	Ratio	0.98 ± 0.33	0.88 ± 0.77	ns[#]
Placenta	Fold change (*Hsd11b1*)	1.00 ± 0.92	1.96 ± 0.84	ns[#]
	Fold change (*Hsd11b2*)	1.00 ± 0.82	1.84 ± 0.55	ns[#]
	Ratio	1.40 ± 1.24	1.15 ± 0.60	ns[#]

(#) Welch's t-test.

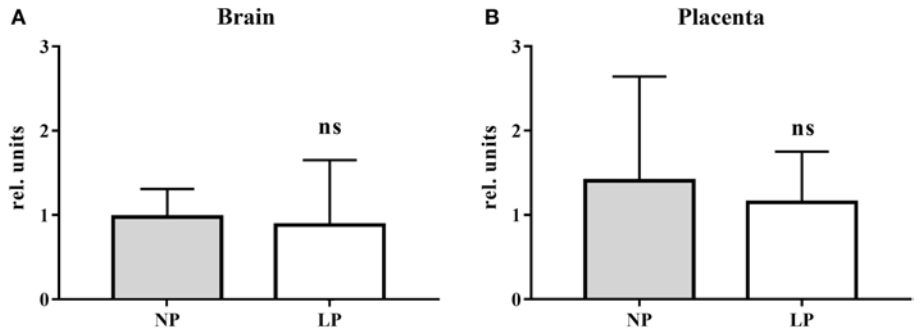

FIGURE 1 | Ratio of *Hsd11b1/2* mRNA expression (qRT-PCR, relative units) in fetal target tissues. **(A)** Fetal rat brain, **(B)** Rat placenta. Legend: Gray column represents NP group ($n = 4$), white column represents LP group ($n = 5$). Legend: rel. units, relative units; ns, not significant.

TABLE 3 | Steroid analysis of maternal whole blood.

Steroids in maternal whole blood (nmol/l)	NP	LP	*p*-value
n	4	5	
Corticosterone (Cort)	1333.00 ± 343.30	1191.00 ± 289.20	ns§
Dehydrocorticosterone (DH-Cort)	91.21 ± 25.23	89.12 ± 34.19	ns#
Ratio (Cort/DH-Cort)	15.32 ± 5.51	14.37 ± 3.71	ns#
Deoxycorticosterone (DOC)	27.63 ± 17.12	14.63 ± 6.49	ns#
Progesterone	761.40 ± 466.50	430.00 ± 177.90	ns§
Testosterone	1.68 ± 0.89	2.21 ± 0.47	ns#

(#)Welch's t-test, (§) Mann–Whitney U-test.

TABLE 4 | Steroid analysis of fetal whole blood.

Steroids in fetal whole blood (nmol/l)	NP	LP	*p*-value
n	4	5	
Corticosterone (Cort)	1740.00 ± 209.90	856.50 ± 177.10	<0.001#
Dehydrocorticosterone (DH-Cort)	218.80 ± 47.30	246.10 ± 46.12	ns#
Ratio (Cort/DH-Cort)	8.10 ± 1.20	3.54 ± 0.73	0.001#
Deoxycorticosterone (DOC)	72.23 ± 16.56	42.54 ± 9.41	0.028#
Progesterone	31.52 ± 4.94	29.38 ± 7.09	ns#

(#) Welch's t-test.

similar profiles of circulating steroids and Cort/DH-Cort-ratios (**Table 3**). In LP fetuses circulating levels of corticosterone were significantly reduced by 50.78% ($p < 0.001$, **Figure 2A**), while significant group differences regarding circulating levels of 11-dehydrocorticosterone were not detected (**Table 4**, **Figure 2A**). The ratio of Cort/DH-Cort was significantly reduced in LP rat fetuses ($p = 0.001$, **Table 4**, **Figure 2B**). As the pools of fetal and maternal glucocorticoids are diaplacentally linked (52, 53), we determined the feto-maternal Cort/DH-Cort-ratio (i.e., normalization of fetal to maternal steroid levels). No significant alteration of fetal Cort/DH-Cort-ratio was found in the LP group compared to NP fetuses (data not shown). Moreover, we found a significant reduction in circulating DOC levels in the LP group ($p = 0.028$, **Table 4**), while progesterone levels were not significantly different.

Subsequently, we examined fetal steroid profiles with regard to sex (**Table 5**). This subgroup analysis revealed no sex-specific differences between NP and LP groups, with regard to circulating steroids.

Steroid Analysis via LC-MS/MS in Fetal Tissue

Brain and placental tissue steroid profiles are listed in **Tables 6**, **7**, respectively. LP diet significantly lowered local corticosterone levels of both brain and placenta by 45.75% ($p = 0.003$, **Figure 3A**) and 45.56% ($p = 0.002$, **Figure 3C**), respectively. In both brain and placenta, levels of 11-dehydrocorticosterone were not significantly altered compared to the NP groups (**Table 6**, **Figure 3A**; **Table 7**, **Figure 3C**, respectively). In the LP group the local Cort/DH-Cort-ratio was significantly lower in fetal brain ($p = 0.016$; **Figure 3B**; **Table 6**) and placenta ($p = 0.014$; **Figure 3D**; **Table 7**) when compared to NP. Furthermore, local DOC levels were significantly reduced in brain ($p = 0.029$, **Table 6**) and placenta ($p = 0.036$, **Table 7**) of LP fetuses. No significant differences of local progesterone in brain and placenta were observed.

In the brain, LP diet significantly reduced local levels of androstenedione and testosterone in males only ($p = 0.011$, $p = 0.025$, respectively; **Supplementary Table S3a**). In contrast to humans (54), the rat placenta synthesizes significant amounts of androgens (androstenedione and testosterone) (55). In our model, local androgen levels of placenta did not significantly differ between NP and LP groups (**Table 7**), even when sex was regarded (**Supplementary Table S3b**). CRH levels were at the detection limit in fetal brain. Subsequently, we were only able to observe a trend to lower CRH in the brain of LP rats ($p = 0.156$, **Table 6**, **Figure 4**). CRH levels in the fetal brain did not show sex-specific differences between NP and LP groups (**Supplementary Table S3a**).

Further examining sex-specific local tissue steroid profiles of brain and placenta between NP and LP

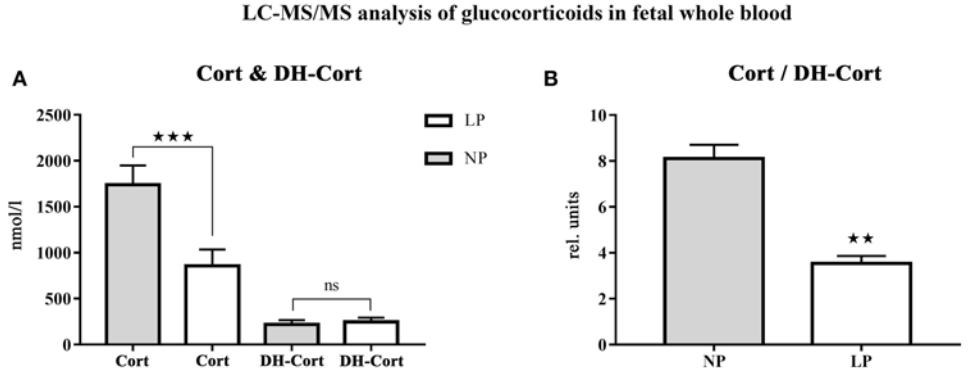

FIGURE 2 | LC-MS/MS analysis of circulating glucocorticoid levels in fetal whole blood. **(A)** Differences of circulating corticosterone (Cort) and 11-dehydrocorticosterone (DH-Cort) levels of fetal NP ($n = 4$) and LP ($n = 5$) whole blood (pmol/g). **(B)** Differences of Cort/DH-Cort -ratio in whole blood. Legend: Gray column represents NP group ($n = 4$), white column represents LP group ($n = 5$), rel. units, relative units; ns, not significant; $^{**}p < 0.01$ and $^{***}p < 0.001$.

TABLE 5 | Sex-specific steroid analysis of fetal whole blood.

	Steroids in fetal whole blood (nmol/l)	NP	LP	*p*-value
	n	4	5	
Male	Corticosterone (Cort)	1803.00 ± 191.20	838.50 ± 152.30	<0.001‡
	Dehydrocorticosterone (DH-Cort)	210.40 ± 56.29	238.50 ± 67.45	ns‡
	Ratio (Cort/DH-Cort)	8.95 ± 2.22	3.71 ± 1.07	<0.001‡
	Deoxycorticosterone (DOC)	73.71 ± 17.00	42.06 ± 14.24	0.014‡
	Progesterone	33.02 ± 4.31	27.27 ± 13.01	ns‡
Female	Corticosterone (Cort)	1677.00 ± 239.00	874.40 ± 218.40	<0.001‡
	Dehydrocorticosterone (DH-Cort)	227.20 ± 47.31	253.70 ± 41.84	ns‡
	Ratio (Cort/DH-Cort)	7.48 ± 0.78	3.49 ± 0.90	0.001‡
	Deoxycorticosterone (DOC)	70.75 ± 16.61	43.02 ± 12.40	0.030‡
	Progesterone	30.01 ± 7.05	31.49 ± 17.89	ns‡

(‡) Two-way Anova.

TABLE 6 | Steroid and CRH analysis of fetal brain.

Steroids and CRH in fetal brain (pmol/g)	NP	LP	*p*-value
n	4	5	
Corticosterone (Cort)	98.86 ± 12.77	53.63 ± 6.09	0.003#
Dehydrocorticosterone (DH-Cort)	104.50 ± 25.86	122.10 ± 37.66	ns#
Ratio (Cort/DH-Cort)	0.99 ± 0.13	0.48 ± 0.10	0.016§
Deoxycorticosterone (DOC)	13.05 ± 2.67	7.68 ± 3.19	0.029#
Progesterone	47.64 ± 7.81	37.86 ± 12.29	ns#
Androstenedione	3.21 ± 0.48	2.35 ± 0.70	ns#
Testosterone	1.87 ± 0.49	1.02 ± 0.81	ns#
CRH (ng/g)	1.50 ± 0.87	0.68 ± 0.31	ns (0.156)#

(#) Welch's t-test, (§) Mann–Whitney U-test.

TABLE 7 | Steroid analysis of placenta.

Steroids in fetal placenta (pmol/g)	NP	LP	*p*-value
n	4	5	
Corticosterone (Cort)	272.80 ± 19.63	148.50 ± 45.76	0.002#
Dehydrocorticosterone (DH-Cort)	309.80 ± 52.00	236.20 ± 43.48	ns#
Ratio (Cort/DH-Cort)	0.93 ± 0.14	0.58 ± 0.18	0.014#
Deoxycorticosterone (DOC)	17.56 ± 4.33	10.17 ± 1.65	0.036#
Progesterone	44.29 ± 12.86	45.89 ± 8.83	ns#
Androstenedione	7.94 ± 1.65	8.58 ± 2.24	ns#
Testosterone	1.09 ± 0.43	0.83 ± 0.25	ns#

(#) Welch's t-test.

groups (**Supplementary Table S2**), we found that local levels of corticosterone in fetal brain and placenta were significantly diminished in both male and female LP rats (**Supplementary Tables S3a,b**, respectively). Furthermore, the sex-specific Cort/DH-Cort-ratio in these organs remained significantly reduced (**Supplementary Table S3**). However, the influence of LP diet on the Cort/DH-Cort-ratio reduction in the female placenta was only a trend ($p = 0.086$, **Supplementary Table S3b**). In the male brain, no difference between NP and LP groups was observed for DOC, while DOC was significantly reduced in female brain ($p = 0.032$, **Supplementary Table S3a**) and in the placenta of both sexes.

Normalization of Tissue LC-MS/MS Results to Body Size

Glucocorticoids and mineralocorticoids are known to significantly influence brain and body weight in rats, respectively (56). As the LP group is characterized by a significant reduction of both body and placental weight (**Table 1**), we normalized local corticosterone, 11-dehydrocorticosterone and deoxycorticosterone levels of brain and placenta in both groups to their respective organ-to-body weight ratio (i.e., steroid x organ weight/body weight, data not shown). Cort/DH-Cort-ratio in brain and placenta of LP fetuses remained

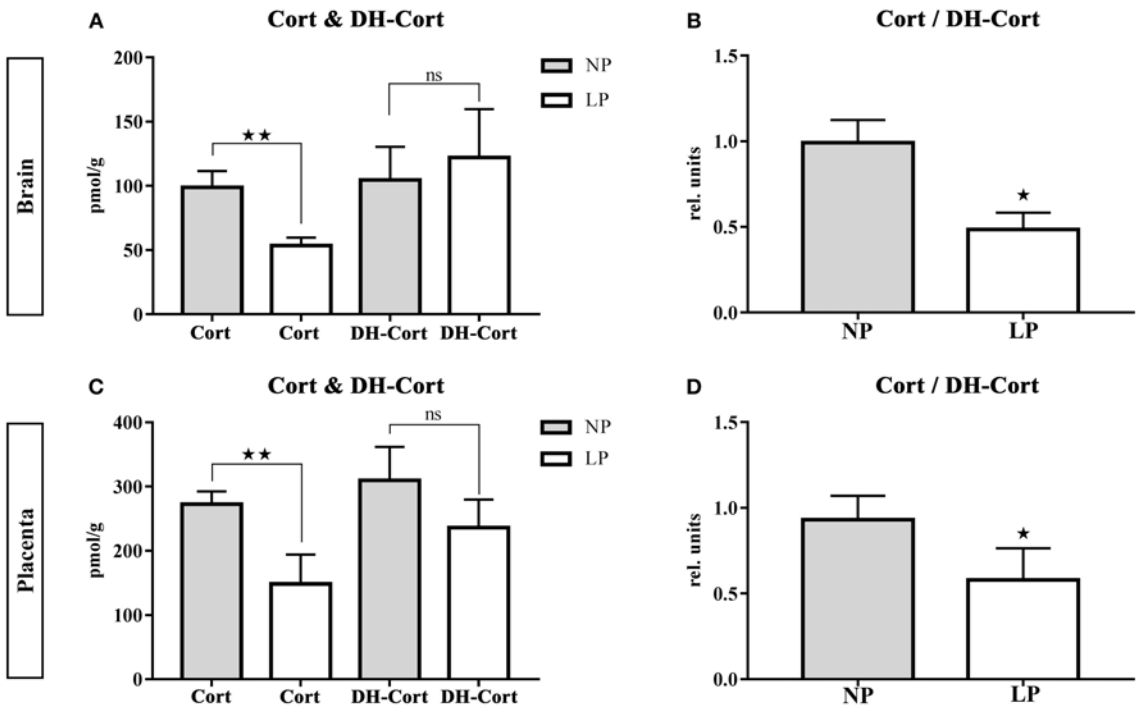

FIGURE 3 | LC-MS/MS analysis of local glucocorticoid levels in fetal target tissues. Results of fetal brain are shown in **(A,B)**, results of placenta are shown in **(C,D)**. **(A)** Differences of corticosterone (Cort) and 11-dehydrocorticosterone (DH-Cort) levels in fetal brains of NP ($n = 4$) and LP ($n = 5$) groups (pmol/g). **(B)** Differences of Cort/DH-Cort -ratio in fetal brain (relative units). **(C)** Differences of corticosterone (Cort) and 11-dehydrocorticosterone (DH-Cort) levels in placenta of NP ($n = 4$) and LP ($n = 5$) groups (pmol/g). **(D)** Differences of Cort/DH-Cort-ratio in placenta of NP ($n = 4$) and LP ($n = 5$) groups (relative units). Legend: Gray column represents NP group ($n = 4$), white column represents LP ($n = 5$) group, rel. units, relative units; ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$.

unaffected, i.e., significantly reduced ($p = 0.016$ and $p = 0.014$, respectively). Moreover, levels of placental corticosterone were significantly reduced ($p = 0.043$). No significance was obtained for deoxycorticosterone. While these results seemed largely independent of sex, the reduction of Cort/DH-Cort-ratio of female placenta remained a trend ($p = 0.086$, data not shown).

Analysis of Steroid Levels Relative to Progesterone

In the rat, progesterone serves as a precursor for mineralocorticoids (DOC) and glucocorticoids (57). Thus, we analyzed the ratio of progesterone to DOC and corticosterone. The results are given in **Supplementary Table S4**. In general, ratios of progesterone to DOC and to corticosterone were increased in all LP specimens tested. However, significance was only reached for progesterone/corticosterone-ratio in whole blood ($p = 0.026$, **Supplementary Table S4**) and progesterone/DOC-ratio in placenta ($p = 0.003$, respectively; **Supplementary Table S4**).

DISCUSSION

Our study analyzed steroid profiles via LC-MS/MS in fetal and maternal whole blood and the corresponding fetal placentas and brains in an established rat model of IUGR by maternal protein restriction. We found that LP nutrition did not alter maternal steroid profiles at E18.5. Furthermore, LP diet did not induce fetal hypercortisolism in our model. Instead, levels of circulating corticosterone were lower in LP litters along with a significant reduction in Cort/DH-Cort-ratio. Circulating DOC levels were reduced in the LP group, while progesterone levels remained constant. These results were independent of sex. Similar to our observation in whole blood, we also found a reduction of fetal corticosterone and Cort/DH-Cort-ratio in our analysis of LP brain and placental steroid profiles.

Effects of Low Protein Diet on Steroid Profiles and CRH of the Fetal Brain

The Cort/DH-Cort-ratio in the brain was largely independent of sex in NP and LP groups. While normalization to organ-to-body weight ratio did not affect Cort/DH-Cort-ratio, the observed reduction in DOC tissue levels seemed secondary to the reduced LP weight development (56). It has been shown by others (56) that levels of corticosterone inversely correlate with brain mass in the rat. Also, there is evidence that administration of antenatal betamethasone might be associated with a reduction in head circumference (22) at term and subsequent impairment of cognitive function in treated infants (20, 21). Thus, a reduced

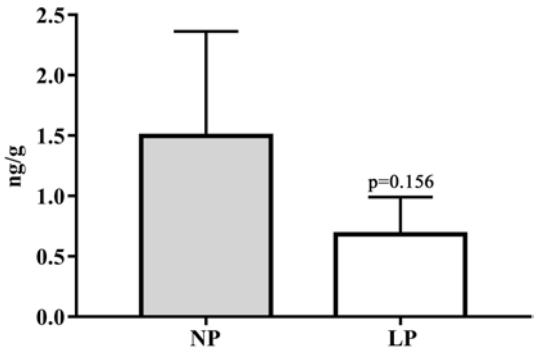

FIGURE 4 | LC-MS/MS analysis of local CRH levels in fetal brain. Differences of CRH of NP ($n = 4$) and LP ($n = 5$) group in brain given in ng/g. Legend: Gray column represents NP ($n = 4$) group, white column represents LP ($n = 5$) group.

Cort/DH-Cort-ratio in our model might argue for a brain sparing effect [reviewed by Miller et al. (58)] in our LP group, as brain weight was not significantly different when compared to NP animals. Brain sparing is associated with asymmetric fetal growth due to increased allocation of nutrients, oxygen, as well as fetal cardiac output to the brain in comparison to other organ systems (58). While this effect promotes survival, it is now apparent that brain sparing might negatively affect brain development in IUGR fetuses (58, 59). Fetal adaptations to adverse intrauterine environment may contribute to an increased postnatal vulnerability for neurodevelopmental and behavioral problems (58, 59). While most animal studies [reviewed by Miller et al. (58)] examined brain sparing effects in rat models of hypoxia or ligation of the uterine artery, García-Contreras et al. (60) examined brain sparing in a malnourished piglet model. They were able to show that nutritional shortage was able to induce local changes in neurotransmitter levels (catecholamines and indoleamines) in swine fetuses, dependent of their sex (60). These transmitters are thought to be related to important cognitive functions (e.g., learning, memory, reward-motivated behavior and stress) (61). To our knowledge, such postnatal behavioral sequelae and neuroendocrine effects have not been studied in our rat model so far. Thus, the potential presence of brain sparing in our LP animals requires future investigation. In line with the observation of sex-specific influences of IUGR on neurotransmitters in swine fetuses (60), we observed differences in brain androgen metabolism (significant reduction of androstenedione and testosterone levels) in our LP male rat fetuses only. These effects might point to brain-specific alterations in local *de novo* synthesis (62) of androgens, as placental androgen levels were not altered by LP diet. It is well-recognized that androgens are important neurodevelopmental factors (63). Androstenedione significantly alters the free fatty acid composition of the fetal brain (64). Excessive levels of androstenedione may induce cellular energy deficits and oxidative stress, potentially driving apoptosis (64). Thus, it might be hypothesized that the reduced androgen levels in the brain of LP males might serve to protect these animals from these adverse effects of IUGR [reviewed by Miller et al. (58)]. Brain compositional changes in free fatty acids warrant further research in our model. The stable androgen levels in the brain of female LP rats could be associated with their ability to metabolize androgens as precursors for estradiol (not determined in this study) (65). Beyond its impact on neurodevelopment, estradiol is known to exert anti-apoptotic effects (66). However, Fernandez-Twinn et al. (39) could only detect an end-gestational rise in circulating estradiol in LP dams of their protein restriction rat model, while no such effect was evident in the fetuses.

Fetal brain CRH levels were close to the lower limit of quantification via LC-MS/MS. Our finding that LP whole brains showed a trend to lower levels of CRH might, however, indicate alterations of superordinate control centers of the HPA-axis. As further targeted histomorphological assessment of these centers (67) is pending in our animal model, we hypothesize that the reduced levels of circulating corticosterone in LP whole blood are associated with reduced CRH in LP brain, despite the lack of significance. A study by Li et al. regarding HPA-axis function in nutritional IUGR of the baboon showed that CRH was the major releasing hormone driving ACTH and cortisol secretion in their model (68). In contrast to our results in the LP rat, the baboon (68), alike other models of maternal nutritional restriction (discussed below), showed an up-regulation of HPA-axis activity. We are currently unaware of other studies examining fetal CRH in the brain of LP rats.

Effects of Low Protein Diet on Placental Hsd11b2 Expression and Function

LP diet led to a significantly reduced placental Cort/DH-Cort-ratio. The reduction of placental corticosterone levels appeared to be independent of organ-to-body weight ratio, similarly to whole blood. While Cort/DH-Cort-reduction did not reach significance in female LP placentas, the Cort/DH-Cort-ratio in whole blood of female LP rats was significantly reduced, alike in male littermates. The lack of significance in female LP placentas might be associated with the small group size. Thus, we concluded that LP diet did not induce fetal hypercortisolism in both sexes in our model.

There is a controversy (39) as to whether intrauterine glucocorticoid exposure is responsible for the reduction of birth weights in the LP model beyond the prevalent deprivation of amino acids (39, 69–71). Our own data does not support this notion. Steroid measurements of whole blood and tissue, via LC-MS/MS, might help to clarify this issue as direct evidence of altered levels of circulating glucocorticoids in the protein restriction model is scarce [ELISA, (39)] and largely based on reports on reduced mRNA expression levels of *Hsd11b2* as a surrogate for enzymatic activity (11, 12, 37, 38).

Jensen Peña et al. recently showed that chronic maternal restraint stress during gestational days 14–20 induced IUGR in Long Evans rats. They observed a significant decrease in placental *Hsd11b2* mRNA expression, associated with an increase in local *Hsd11b2* DNA methylation (40). Accordingly, fetal overexposure to maternal corticosterone was assumed to be

involved in the pathogenesis of IUGR. However, no local or circulating glucocorticoid levels were determined in this study. Interestingly, while *Hsd11b2* mRNA levels in the brain remained unaffected (40), the DNA methylation patterns within the *Hsd11b2* promoter were similar to the ones determined in placenta, indicating a certain neuroplacental correlation (40).

In our model, we did not detect significant expressional changes of *Hsd11b1* and *2* mRNA in placenta. Our tissue LC-MS/MS method allows for the direct analysis of local steroid metabolism. Based on the observed dichotomy between our qRT-PCR and LC-MS/MS, we hypothesize that the latter method might be superior regarding the detection sensitivity of alterations in glucocorticoid metabolism. Interestingly, others have just recently found great variability in multiple mRNA species associated with cortisol production, metabolism, and action (72).

Rat Models of Intrauterine Malnutrition and the Role of Glucocorticoids

The role of fetal overexposure to glucocorticoids in IUGR animal models of nutritional restriction remains somewhat unclear, partly due to the differences in feeding, and analytical methods used: employing a rat model where dams were kept on a restricted diet in late gestation (50% of their daily *ad libitum* intake), Blondeau et al. found increased maternal and fetal levels of corticosterone using radio-immunoassays (73). Fetuses had low birth weight and significantly reduced pancreatic insulin content. Thus, an inverse correlation of circulating maternal glucocorticoid levels with fetal β-cell mass was hypothesized (73). Similarly, other studies involving maternal undernutrition (either 50% or protein restriction) showed an increase in maternal levels of corticosterone and a reduction of placental *Hsd11b2* in rats (11, 13).

In contrast, Fernandez-Twinn et al. found serum corticosterone (ELISA detection) in both maternal and fetal circulation (39) to be unaffected by maternal protein restriction using a Wistar rat model of isoenergetic protein restriction throughout gestation]80 g protein/kg vs. 200 g protein/kg (74), *ad libitum*]. However, they were able to identify endocrine changes in their dams (hyperglycemia with concomitant hyperinsulinism, increased levels of prolactin and decreased progesterone levels in early pregnancy; compensatory elevated oestradiol levels and reduced leptin levels in late pregnancy), that might contribute to the programming of poor glucose tolerance, insulin resistance, and the metabolic syndrome in their animals (39). By then, glucocorticoid levels had not been studied in the rat protein restriction model. Hence, based on these findings, Fernandez-Twinn et al. concluded that glucocorticoid levels may not have played a major role in direct programming of eventual adult disease in the developing fetus of their model of protein restriction (39).

Our model of protein restriction has not been characterized to develop postnatal disturbance of glucose tolerance or diabetes in young animals (75) and we have found a significant reduction of corticosterone in our LP rats. Thus, we hypothesize, that the hyperglycemic metabolic state of their dams (39) might have masked potentially reduced glucocorticoid levels so that corticosterone levels presumably matched those found in their NP controls. Exaggerated glucocorticoid secretion has been described to be present in diabetic animals and patients (76). Etiopathologically, the observed maternal hyperglycemia might have been due to the fact that their protein restriction model (74) mainly used monosaccharides to achieve isocaloric energy supplementation (39) and that *ad libitum* fed LP dams further showed an increased daily food intake (39). In contrast, such an effect did not occur in our model (daily food intake 25 g) and isocaloric conditions were fostered by increased substitution of polysaccharides and fat. Also, we observed an increase of the placenta-to-body weight ratio at E18.5, while Fernandez-Twinn et al. observed the ratio to be unchanged (E18), or rather reduced (E21) (39). This might suggest a higher placental capacity for the compensation of maternal protein restriction in our model.

In light of the presented results, one might hypothesize that the maternal food restriction model (i.e., 50% total intake restriction) might more closely resemble models of intrauterine stress than our model of protein restriction, as it leads to increased fetal glucocorticoids. Conclusions drawn from comparison of our study with the only other protein restriction model that examined fetal corticosterone levels (39) seem limited by the above-mentioned maternal metabolic differences. However, corticosterone levels of LP fetuses never exceeded NP levels in both studies. In line with this finding, our previous work (77) indicates that adult male LP rats did not show changes in their circulating glucocorticoid levels, postnatally.

Potential Role of Progesterone in Energy Homeostasis

Progesterone is an essential neuroactive steroid during gestation (78). It is synthesized in maternal tissue, placenta, and the fetal brain, the latter of which is the major target of the steroid (79). Increased local biosynthesis of progesterone and its derivatives may contribute to adaptive processes in maternal and fetal brain during pregnancy (80). Progesterone also exerts neuroprotective functions (78).

While others found progesterone levels of protein restricted dams to be temporarily reduced, we did not detect an influence of LP diet on maternal and fetal progesterone levels at E18.5, despite a reduction in corticosterone and Cort/DH-Cort-ratio in placenta, fetal whole blood, and fetal brain. Thus, it might be speculated that the observed reduction of active glucocorticoids in our LP animals could have resulted, in part, from an organismal effort to maintain constant progesterone levels. Based on our findings, it can be hypothesized that glucocorticoid metabolism is reduced in favor of progesterone maintenance in our LP rats. Interestingly, progesterone is also known to enhance amino acid utilization by the liver (81, 82), which could be of relevance for energy homeostasis in our LP rats.

Limitations

The presented results are limited by the small power of our study. Thus, some of our findings, especially of sex-related differences, represent trends and have to be interpreted with care. The expression of *Hsd11b 1* and *2* was determined on the mRNA

level only, however, it remains elusive how mRNA expression translates to protein levels and enzymatic activity in target tissues. While LC-MS/MS has already served as a valid tool for the investigation of glucocorticoid turnover rates in tissue (9), we lacked sufficient amounts of material to perform microsomal enzymatic activity analyses in our study. Furthermore, as only a single time-point of gestation was examined, so far, dynamics in steroid metabolism during pregnancy remain undetermined. It needs to be noted, that the displayed results of our work are only focused on the analysis of fetuses from the proximal uterine horn, which represents the site with the highest maternal blood supply (13). Methodologically, we have avoided brain perfusion prior to LC-MS/MS tissue analysis, as blood contamination has been shown to have little effect on brain steroid levels and saline perfusion may significantly alter local steroid levels (47). Furthermore, we used anesthesia by isoflurane before sacrifice in all animals. While this technique avoids induction of glucocorticoids caused by the injection of anesthetic compounds (83), Bekhbat et al. have recently shown that the use of isoflurane might sex-specifically alter corticosterone levels as determined via ELISA (84). As we currently cannot rule out artifacts in response to collection paradigms, caution has to be used when transferring the presented data to models with different anesthesia.

CONCLUSION

LP diet induced IUGR in our rats, yet it did not impair placental Hsd11b2 gatekeeping function in our study. We did not observe fetal hypercortisolism, as described by other rat models of IUGR (especially general undernutrition and stress models). In contrast, fetal corticosterone levels were significantly reduced in the LP group. This finding seemed independent of sex and was evident in whole blood, placenta and fetal brain. We hypothesize that our LP model does not represent a genuine model of intrauterine stress. Accordingly, our model might not be appropriate to study mechanisms involved in modulating fetal exposure to maternal steroids in IUGR. Thus, while the above-mentioned IUGR models imply that alterations of neuroplacental glucocorticoid cross-talk predispose for neurodevelopmental sequelae, this mechanism might not be present in our LP model. Our maternal protein restriction model may be seen as an equivalent to maternal protein malnutrition in developing countries (85) and might aid in understanding of mechanisms involved in fetal development under protein restriction conditions and the associated postnatal sequelae [especially reno-vascular and cardiac (3, 4, 46, 86)]. However, these may not be significant to and/or representative of mechanisms involved in intrauterine stress in general. At this time we cannot exclude that fetal hypercortisolism was preexistent at earlier gestational stages and that our analysis only determined a certain fatigue of HPA-axis function at the end of pregnancy. Nevertheless, the observed endocrine traits could suggest fetal efforts to achieve anabolic conditions, e.g., brain sparing, in the state of amino acid shortage. If such an effect was present in LP fetuses, it might negatively affect neurodevelopment. Our future studies will, therefore, include additional analysis of postnatal behavior, glucocorticoid levels, tissue metabolomics, and longitudinal steroid profiling beyond corticosterone, e.g., of progesterone derivatives.

DATA AVAILABILITY

All relevant datasets for this study are included in the manuscript and the supplementary files. The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AH and FF designed the experiments and supervised the study. MS, RW, NC, and FF conducted and interpreted the experiments. MS, MCS, FF, and MRa performed the data analysis. MS, FF, and MRa wrote the manuscript. FF, MCS, and MS participated in the statistical analysis. WR, CM-C, AH, HH, MCS, and MRu critically revised the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of I. Allabauer and K. Heussner at the research facility of the Department of Pediatrics and Adolescent Medicine, Friedrich-Alexander University Erlangen-Nuremberg, Erlangen, Germany. Animal procedures, endocrine analysis and respective data acquisition were performed by MS in fulfillment of the requirements for obtaining the degree Dr. med. at the Friedrich-Alexander-University of Erlangen-Nuremberg, Department of Pediatrics and Adolescent Medicine, Germany. The authors kindly thank S. Fahlbusch, Kirkland, USA for proofreading and language editing. We acknowledge support by Deutsche Forschungsgemeinschaft and Friedrich-Alexander-University Erlangen-Nürnberg (FAU) within the funding programme Open Access Publishing.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00124/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 20 March 2019
doi: 10.3389/fendo.2019.00160

Failure of Decidualization and Maternal Immune Tolerance Underlies Uterovascular Resistance in Intra Uterine Growth Restriction

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OPEN ACCESS

Edited by:
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Reviewed by:
Alexander Beristain, University of British Columbia, Canada
Ana Cecilia Mestre Citrinovitz, Heidelberg University Hospital, Germany

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *26 October 2018*
Accepted: *25 February 2019*
Published: *20 March 2019*

Citation:
Dunk C, Kwan M, Hazan A, Walker S, Wright JK, Harris LK, Jones RL, Keating S, Kingdom JCP, Whittle W, Maxwell C and Lye SJ (2019) Failure of Decidualization and Maternal Immune Tolerance Underlies Uterovascular Resistance in Intra Uterine Growth Restriction. Front. Endocrinol. 10:160. doi: 10.3389/fendo.2019.00160

Failure of uterine vascular transformation is associated with pregnancy complications including Intra Uterine Growth Restriction (IUGR). The decidua and its immune cell populations play a key role in the earliest stages of this process. Here we investigate the hypothesis that abnormal decidualization and failure of maternal immune tolerance in the second trimester may underlie the uteroplacental pathology of IUGR. Placental bed biopsies were obtained from women undergoing elective caesarian delivery of a healthy term pregnancy, an IUGR pregnancy or a pregnancy complicated by both IUGR and preeclampsia. Decidual tissues were also collected from second trimester terminations from women with either normal or high uterine artery Doppler pulsatile index (PI). Immunohistochemical image analysis and flow cytometry were used to quantify vascular remodeling, decidual leukocytes and decidual status in cases vs. controls. Biopsies from pregnancies complicated by severe IUGR with a high uterine artery pulsatile index (PI) displayed a lack of: myometrial vascular transformation, interstitial, and endovascular extravillous trophoblast (EVT) invasion, and a lower number of maternal leukocytes. Apoptotic mural EVT were observed in association with mature dendritic cells and T cells in the IUGR samples. Second trimester pregnancies with high uterine artery PI displayed a higher incidence of small for gestational age fetuses; a skewed decidual immunology with higher numbers of; CD8 T cells, mature CD83 dendritic cells and lymphatic vessels that were packed with decidual leukocytes. The decidual stromal cells (DSCs) failed to differentiate into the large secretory DSC in these cases, remaining small and cuboidal and expressing lower levels of the nuclear progesterone receptor isoform B, and DSC markers Insulin Growth Factor Binding protein-1 (IGFBP-1) and CD10 as compared to controls. This study shows that defective progesterone mediated decidualization and a hostile maternal immune response against the invading endovascular EVT contribute to the failure of uterovascular remodeling in IUGR pregnancies.

Keywords: IUGR, uterovascular transformation, decidua, EVT, immunology, T cells, dendritic, progesterone

INTRODUCTION

Healthy pregnancies display uterine spiral arteries where the endothelial layer has been replaced with placentally-derived EVT and the muscular and elastic layers have been replaced with fibrinoid matrix. This results in their transformation from narrow, vasoactive arteries to flaccid, maximally dilated vessels unresponsive to vasomotor stimuli, and is necessary in order to increase maternal blood flow to the intervillous space for optimal fetal growth (1). Failed transformation of the spiral arteries in contrast is postulated as a contributing cause to several disorders of pregnancy including preeclampsia (PE), intrauterine growth restriction (IUGR), spontaneous preterm labor, preterm premature rupture of the membranes, and miscarriage (2–6). These cases are characterized by reduced utero-placental blood flow, documented by the observation of persistent high resistance waveforms using color/pulsed Doppler ultrasound of the proximal uterine spiral arteries (6–8). Such women do not exhibit any 2nd trimester fall in blood pressure (9) and placental bed biopsies (PBBx) from these subjects at delivery show failure of EVT invasion into the myometrial portions of spiral arteries despite adequate decidual interstitial EVT invasion (10–12). Histologic findings in these pathological PBBx represent varying combinations of the following features—collectively termed as decidual vasculopathy—on hematoxylin eosin stained sections; persistent muscularization of the spiral artery segments, a lack of endovascular trophoblast; apoptosis of endovascular EVT; a maternal leukocyte infiltrate, capable of inducing trophoblast apoptosis; atherosis and further narrowing of the vessel lumens or thrombosis in the spiral artery(2, 3, 5, 13–15). To date most studies have focused on the uteroplacental pathology of PE pregnancies while less is known about the uteroplacental pathology of IUGR. It is also unknown if this failure of uterovascular remodeling is primarily due to an EVT defect or in fact to a defect of decidualization. Recently, Brosens et al. have proposed that defective differentiation of the secretory endometrium to the decidua in the earliest stages of pregnancy may be a significant contributor to the development of the Great Obstetrical Conditions (16, 17).

Human decidualization occurs independently of pregnancy and is initiated following ovulation every menstrual cycle. In the mid-late secretory phase, the actions of progesterone, estradiol, and cAMP coordinate to promote the differentiation of the estrogen-primed endometrium into a receptive tissue prepared for blastocyst implantation in the event of fertilization. This process is referred to as decidualization and requires the coordination of a number of key events including; the differentiation of fibroblast-like endometrial stromal cells into large, rounded, secretory decidual stromal cells (DSC), the growth and elongation of the uterine spiral arteries, and the recruitment and specialization of a unique angiogenic decidual immune population (18, 19). The decidual leukocytes rapidly increase in number during the late secretory period to the effect that they comprise up to 40% of all decidual cells in the first trimester of pregnancy (20, 21). They are recruited from the periphery by chemokines secreted by the differentiated DSC, or proliferate within the decidua as it differentiates (22–24). The leukocyte populations include uterine natural killer cells (uNK, 50–70%), macrophage (20%), T-cells (10%), and myeloid dendritic cells (2%) and play a critical role in the development and transformation of the uterine spiral arteries (25–28).

Following fertilization and blastocyst attachment utero vascular remodeling is initiated early in gestation in both decidual and myometrial junctional zone segments of the spiral arteries. These changes occur prior to the presence of trophoblast in the tissue and are associated with disorganization of the perivascular smooth muscle cells (VSMC) layers, and coincide with the presence of large numbers of maternal leukocytes within the decidua (29–31). In decidual vessels undergoing active transformation numerous perivascular uNK and macrophages are found in close association with and infiltrating the disrupted VSMC layers both *in vivo* and *in vitro* (32–34). We have recently shown that these leukocytes secrete matrix metalloproteinases 2,−7,−9,−11,−16, and−19 which they utilize to disrupt ECM of the vascular wall (31, 35, 36). This leads to separation and disorganization of the VSMC layers and ultimately dedifferentiation and death of the VSMC (34, 37, 38).Vessels newly dilated by trophoblast-independent remodeling are further transformed and stabilized through trophoblast-dependent vascular remodeling, through which maximal dilation of the spiral arteries is finally achieved (39). We have suggested that the influx of decidual leukocytes into the vessel wall may also provide a chemokine stimulus to draw endovascular EVT up the vascular lumen to mediate the last stabilization of the transformed artery (19, 40). It has been shown that uNK secrete CCL8, CXCL-10, and CCL5 to promote EVT invasion via CXCR1 and CXCR3 receptors (40). Interestingly numbers of uNK are reduced in the decidua of term IUGR pregnancies (41), suggesting that altered uNK-EVT interactions may contribute to the failure of endovascular invasion associated with uteroplacental pathology.

The importance of leukocytes in the uterine vascular remodeling of the first trimester is well-established, yet new functions mediated by specific leukocyte populations and interactions between different decidual leukocyte populations and EVT are still being discovered (42). However, less is known about the second trimester decidual leukocyte populations although uterovascular transformation continues well into the 2nd trimester (43). In general studies support the development of a Th2 dominant tolerogenic immune environment in the second trimester under the control of rising levels of placental progesterone (44). Mechanisms employed by the various cell populations, including the DSC, act primarily to either reduce dNK cytotoxicity, or prevent activation of T-cell mediate immune responses either directly or indirectly by altering the phenotypes of antigen-presenting cells (macrophage and dendritic cells) (45, 46). We have previously shown that in the second trimester decidua macrophage differentiate to an alternate M2c proangiogenic tissue remodeling phenotype, T cells increase and are dominated by CD4 T helper cells and T-reg, and dendritic cells are maintained in an immature phenotype (27).

In this study we investigated the hypothesis that decidualization would be compromised in the 2nd trimester of pregnancies from women displaying high uterine artery pulsatile

index (PI) and that this would be identified by both disturbances in the development, recruitment, and adaptation of the decidual leukocyte populations and decidualization of the DSC. We focused our investigation on 2nd trimester pregnancies carrying small for gestational age (SGA) fetuses in an aim to inform our observations in the 3rd trimester placental bed biopsies from IUGR pregnancies and thus contribute to the understanding of the development of uterovascular pathology in these cases.

METHODS

Tissue Collection

This study was carried out in accordance with the recommendations of Mount Sinai Hospital Research Ethics Board, Sinai Health System. The protocol was approved by the Mount Sinai Hospital Research Ethics Board, Sinai Health System REB# 02-0061A and 12-0007E]. All subjects gave written informed consent in accordance with the Declaration of Helsinki. All research using human tissues was performed in a class II certified laboratory by qualified staff trained in biological and chemical safety protocols, and in accordance with Health Canada guidelines and regulations.

Placental bed biopsies were collected from healthy women undergoing elective caesarian section at term ($n = 15$) and from women with high uterine artery Doppler pulsatile index (PI > 1.5) undergoing caesarian section and carrying a IUGR fetus <10% expected fetal weight ($n = 8$). Five women with preeclampsia, a high uterine artery PI (>1.5) and carrying an IUGR fetus were also recruited for comparison. PI is calculated from measurement of (systolic velocity-diastolic velocity)/mean velocity of the uterine or umbilical artery. Biopsies were collected immediately following delivery of the fetus and placenta under direct visualization via the introduction of the punch biopsy forceps through the uterine incision. Three biopsies were collected; two from the placental site and one from the opposing wall of the uterus. Patient demographic data is presented in **Table 1**; placental pathology is presented in **Table 2**.

Decidual tissue was obtained from healthy women undergoing elective termination for medical reasons between 14 and 19 weeks of gestation at the Second Trimester Interruption of Pregnancy (STIPS) clinic. Second trimester tissues were obtained via dilation and evacuation 24 h after insertion of a cervical laminaria. Uterine artery Doppler assessment was performed prior to the termination procedure. Of the second trimester cases collected, 6 had normal uterine artery Doppler and 6 had elevated uterine artery Doppler (PI > 1.6). Fetal size was also measured by ultrasound (**Table 3**). The increase in the PI cut-off in this group is to account for the higher uterine artery PI normally observed at this gestational age (47).

TABLE 1 | Patient demographics of the placental bed biopsy study.

Diagnosis	Control ($n = 15$)	IUGR ($n = 8$)	IUGR and PE ($n = 5$)
No. of primigravid	2	1	0
Race (% white)	60%	50%	20%*
Umbilical artery Doppler	Normal, 15	AEDV, 3 REDV, 3 Increased PI, 2	AEDV, 4 Increased PI, 1
Uterine artery Doppler	Normal, 15	Increased PI, 7 Abnormal, 5 Not recorded, 1	Increased PI, 5 Abnormal, 1
No. of vessels examined	Decidual (44) Myometrial (56)	Decidual (21) Myometrial (19)	Decidual (12) Myometrial (13)
Maternal age, (year)	36 (4.3)	37.4 (4.7)	31.6 (5.1)
Systolic BP,(mm Hg)	104.07 (31.05)	120.0 (10.43)	148.80 (35.46)*
Diastolic BP, (mm Hg)	68.71 (11.86)	78.38 (8.42)	91.60 (11.08)*
Gestational age, week	>37, 15	<34, 6* >37, 2	< 34, 4* >37, 1
Birth weight, kg	3.3 (0.52)	1.30 (0.43)*	1.17 (0.36)*
Birth weight percentile	>50%, 15	5th−10th, 6 <5th, 2	5th−10th, 1 <5th, 4
Proteinuria	Abnormal 1, Not recorded, 14	Abnormal, 1 Not recorded, 8	Abnormal, 4 Not recorded, 1

*A/REDV, Absent/reduced endovascular dopplar velocity. BP, blood pressure *P < 0.05 compared to healthy term controls.*

TABLE 2 | Placental histopathology in cases and controls.

Placental pathology	Normal ($n = 15$)	IUGR ($n = 8$)
Small placenta <25%	0	5
Distal villous hypoplasia	0	2
Fetal thrombotic vasculopathy	1	3
Advanced villous maturity	0	3
Decidual vasculopathy	0	2
Multifocal infarction	0	3
>1 significant lesion	0	2
Villitis of unknown etiology	0	2
Umbilical cord abnormalities	1	4

Immunohistochemistry and Image Analysis

Serial 5 μm sections of each biopsy were immunostained using an avidin-biotin peroxidase technique as previously described (32). Antigen retrieval was performed by either heat mediated 10 mM sodium citrate (pH 6) solution or 1 mM EDTA (pH9) as determined by optimization studies and is indicated in **Table 4**. Blocking was performed for 1 h using Dako Protein Serum-Free Blocking Solution (Dako). Slides were then incubated with primary antibodies to identify EVT (CK7 and HLAG), myometrial and VSMC (Smooth muscle actin), endothelial cells (CD31), and decidual leukocytes (CD45) diluted in PBS or negative IgG controls (specific to the species in which primary antibodies were raised) and incubated overnight at 4°C. Further leukocyte subsets were investigated using antibodies against: CD56 (uNK), CD68 (macrophage), CD3, CD4, and CD8 (T Cells) and CD209 and CD83 (immature and mature dendritic cells). Antibodies are detailed in **Table 4**. Following incubation with species appropriate secondary biotinylated antibodies slides were washed with PBS, developed using the Liquid DAB+ (3,3-diaminobenzidine) Chromogen System (Dako), and counterstained with diluted Gill's No. 1 Hematoxylin

TABLE 3 | Patient demographics of the 2nd trimester decidua study.

Diagnosis	Control ($n = 6$)	High Ut A PI ($n = 6$)
Gestational age, week	14–19	17–19
Uterine artery Doppler	Normal, 6	Increased PI, 6 Abnormal, 5
No. of vessels examined	Decidual (22)	Decidual (34)
Maternal age, y	33 (2.3)	35.4 (3.1)
Systolic BP, mm Hg	119.23 (12.9)	120.0 (5.52)
Diastolic BP, mm Hg	76.71 (7.83)	80.12 (5.47)
Small for gestational age	0	4
FETAL ABNORMALITIES:		
Chromosomal	2	2
Genetic	2	1
Hydrops	1	0
Limb abnormalities	1	1
No heart beat	1	1
Severe IUGR	0	1

(Sigma-Aldrich, Oakville, ON). True PBBx were confirmed by the presence of myometrium, decidua, one or more uterine spiral arteries, and positive staining for HLA-G or CK7 (indicating trophoblast presence). For negative controls, the specific primary antibody was replaced by normal mouse or rabbit IgG at the same antibody concentration used to stain test samples. No staining of the negative controls was observed. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using the in situ cell death detection kit-POD and according to the manufacturer's instructions (R and D systems, Oakville, Canada).

Analysis of Structural Alterations in the Placental Bed

Structural alternations in the placental bed biopsies were scored based on previous publications (14, 48). Sections were scored by 2 observers (C.D. and S.K.) blinded to the sections identity and showed good agreement between observers. Decidual and myometrial vessels were considered separately. Disruption of the vessel wall was graded into 3 groups (preserved, disorganized (disorganization and some loss of the media) and absent/grossly disorganized (little or no media remains). Interstitial EVT (inEVT) found $> 50\,\mu m$ from the vessel wall were graded as absent (0), low density (1) or high density (2). Intramural EVT (mEVT) and endovascular EVT (enEVT) were scored as present or absent if within the vessel media, or where it once was, or lumen, respectively.

Image Analysis and Quantification

Quantification of leukocyte subtypes and decidual markers in the PBBx samples controls ($n = 15$ basalis, $n = 15$ paretalis) and IUGR ($n = 8$) and IUGR with maternal PE ($n = 5$) or second trimester human decidual samples from pregnancies with high uterine artery PI ($n = 6$) or normal uterine artery PI ($n = 6$) was performed using an Olympus BX61 microscope equipped with an Olympus DP72 camera, and the newCAST software (Visopharm). Firstly 2 separate masks were established covering the decidual and myometrial areas of the PBBx section. Counts were performed using a standard protocol that assigned random counting frames covering 5% of each total masked tissue area. Brown, positively-staining cells and blue, negatively-staining cells (haematoxylin-stained) were counted at 10X magnification. A positively stained ratio was generated by dividing the total numbers of brown, positively-staining cells by the total number of cells counted in the tissue area).

Isolation of Decidual Leukocytes

Decidual tissue was processed as described in Kwan et al. (27). Briefly, decidua was washed and minced finely and flushed with $HBSS^{+/+}$ 2 to 3 times to ensure maximal release of leukocytes. Decidual cells were collected via filtration through 100 and 70 μm sieves and centrifugation (700 g for 10 min at 4°C). Isolated cells were suspended in RPMI-1640 + 10% FBS and fibroblasts eliminated through differential attachment to tissue culture plates (37°C for 20 min). Following incubation, remaining cells were passed through a 40 μm filter and incubated in erythrocyte lysis buffer EL (Qiagen, Toronto, ON, Canada) for 20 min at 4°C. The final cell suspension was incubated in serum-free protein block (Dako, Burlington, ON, Canada) for 1 h on ice and diluted to a final concentration of 10^6cells/mL.

Cell Labeling and FACS Analysis Gating Strategy

Isolated cells were incubated with fluorochrome-conjugated mouse monoclonal antibodies in 200 μl staining volume for 45 min at 4°C in the dark. Cells were washed in PBS and re-suspended in 200 μl stabilizing-fixative permeabilization buffer (BD Biosciences, Mississauga, ON, Canada) to prevent dissociation of tandem dyes and analyzed using a FACSAria flow cytometer (BD Biosciences, Mississauga, ON, Canada) and FlowJo software (Tree Star, Ashland, OR, USA). Cell debris and aggregates were excluded by gating on forward vs. side scatter, then by height vs. width of forward and side scatter plots prior to population discrimination. Non-leukocyte events were excluded from analysis using threshold gates set on CD45 fluorescence. Positive leukocyte subpopulations were identified upon comparison of fully-stained samples to fluorescence minus one (FMO) and isotype controls. Dendritic cell populations were assessed in more detail within the high side scatter gate within the CD45 positive leukocyte population. Antibodies used are listed in **Table 4**.

Statistical Analysis

Outliers were identified using Grubb's test and excluded from subsequent analysis. Prism 5 (GraphPad, La Jolla, CA, USA) was used to perform ANOVA or unpaired Student's *t*-tests assessing changes in means ± standard deviation of the mean (SD) of patient demographics compared to control term pregnancies and leukocyte populations between second trimester normal and high uterine artery PI cases. Data was tested for normality prior to ANOVA analysis Leukocyte populations were quantified as a percentage of total $CD45^+$ leukocytes except where specified; $p < 0.05$ or greater was considered statistically significant.

TABLE 4 | Flow cytometry and immunohistochemistry antibody information.

Antibody	Clone	Company (cat no)	Dilution/Ag Retrieval	Specificity
CD45 APC-Cy7	2D1	BD 557833	1:33	Common Leukocyte Antigen
CD3 FITC	HIT3a	BD 555339	1:25	T Lymphocytes
CD56 PE-Cy7	B159	BD 557747	1:33	Uterine Natural Killer Cells (NCAM)
CD68 PE	Y1-82A	BD 555743	1:6.6	Mac-1 Integrin (Activation Marker)
CD209 PerCP-Cy5.5	DCN-46	BD 558263	1:10	Immature Dendritic Cells (DC)
CD205 PE	MG38	BD 558069	1:25	Intermediate/Antigen Uptake Receptor DC
CD83 APC	HB15e	BD 551073	1:5	Mature Antigen Presentation DC
CK-7	OV-TL 12/30	Dako M7018	1:200 1 mM EDTA pH9. Heat	Epithelial Cells
HLA-G	4H84	Exbio 11-499-C	1:300 10 mM Na Citrate pH6. Heat	Extravillous Trophoblast
SMA	1A4	Dako M0851	1:100 10 mM Na Citrate pH6. Heat	Smooth Muscle Cells
CD31	JC70A	Dako M0823	1:50 10 mM Na Citrate pH6. Heat	Endothelial Cells
CD45	2B11+PD7/26	Dako M0701	1:100 10 mM Na Citrate pH6. Heat	Common Leukocyte Antigen
CD56/NCAM	123C3	Dako M7204	1:50 1 mM EDTA pH9. Heat	Uterine Natural Killer Cells
CD68	PG-M1	Dako M0876	1:200 10 mM Na Citrate pH6. Heat	Macrophage
CD3	F7.2.38	Dako M7254	1:50 1 mM EDTA pH9.Heat	T cells
CD4	EPR6855	Abcam Ab133616	1:200 10 mM Na Citrate pH6. Heat	T helper cells
CD8	EP1150Y	Abcam Ab93278	1:200 10 mM Na Citrate pH6. Heat	Cytotoxic T cells
CD209	120612	R and D systems MAB1621	1:200 1 mM EDTA pH9. Heat	Immature DC
CD83	HB15e	E Bioscience 14-0839-82	1:50 10 mM Na Citrate pH6. Heat	Mature Antigen Presentation DC
D240/podoplanin	730-01	Covance SIG-3730	1:50 1 mM EDTA pH9. Heat	Lymphatic endothelium
IGFBP-1	H-120	SC Biotech sc-13097	1:200 10 mM Na Citrate pH6 Heat	IGF binding protein 1
CD10	97C5	SC Biotech sc-19993	1:50 1 mM EDTA pH9. Heat	Common acute lymphocytic leukemia antigen
Mouse IgG	DAK-501	Dako X0931	1:300	Mouse Fc
Rabbit IgG	Rabbit Ig fraction	Dako X0903	1:300	Rabbit Fc

RESULTS

Failure of Uterine Vascular Transformation Is Associated With Apoptotic EVT and Altered Maternal Immune Status in IUGR Placental Bed Biopsies

In comparison to healthy term pregnancies in the control group (Normal, $n = 15$) the idiopathic IUGR patient group had; significantly higher uterine artery pulsatile indices (PI), absent (AEDV) or reversed (REDV) umbilical end-diastolic velocity in their umbilical artery, babies born at <10th %tile of weight for gestational age, and placental pathology indicative of IUGR ($n = 8$). A further 5 cases of severe IUGR were collected from women with preeclampsia defined according to the ACOG criteria, showing elevated maternal blood pressure, proteinuria and indicators of end organ damage (**Tables 1**, **2**).

Uterine spiral arteries in the myometrial decidual junction of PBBx from healthy term pregnancies displayed significant disorganization and complete disruption of their vascular smooth muscle (VSMC) media and loss of the endothelium, associated with the presence of both enEVT and inEVT (**Figure 1**, Patient 1). Full transformation was associated with

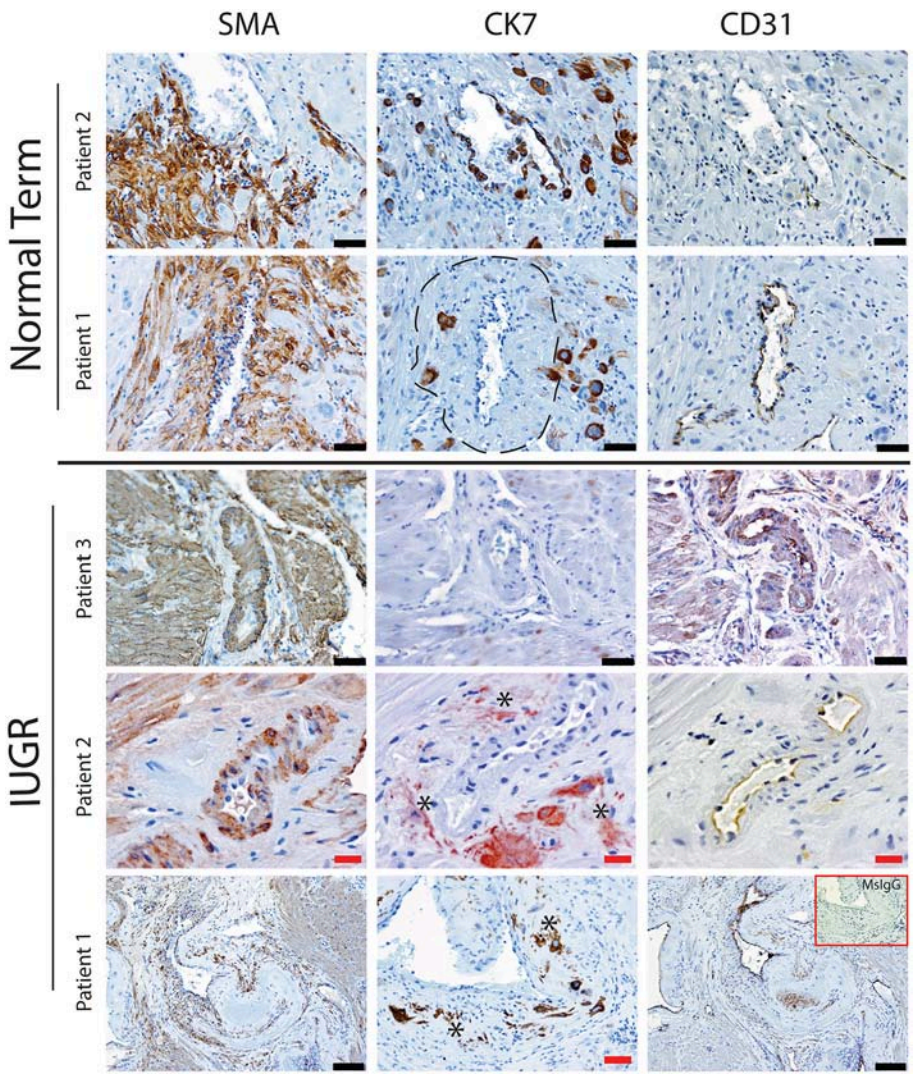

FIGURE 1 | Failed uterovascular transformation in IUGR is associated with persistance of vascular media and failure of interstitial and endovascular invasion. Immunohistochemical staining was performed to determine the degree of uterine artery transformation in PBBx biopsies using antibodies against smooth muscle actin (SMA, Column 1) cytokeratin 7 (CK7, Column 2) and PECAM (CD31, Column 3). Representative serial sections of control ($n = 2/15$) and IUGR PBBx ($n = 3/8$) uterine spiral arteries at the decidual myometrial junction are shown. Asterisks denote the presence of fragmented likely apopototic mural EVT in the IUGR cases. Fibrin deposition in the fully transformed artery is shown in patient 1 Normal marked by the hatched line. Negative control mouse IgG1 is shown in the inset lower right. Scale bars: black = 50 μM, 100x magnification; red = 20 μM, 200x.magnification.

mEVT and fibrin deposition in the arterial media (marked area, Patient 2). In contrast, and as expected, PBBx from IUGR pregnancies showed a higher number of preserved uterine arteries with thick organized vascular smooth muscle media and intact endothelium (**Figure 1,** Patients 1–3). Quantitation of all myometrial vessels across the patient subgroups showed that showed that the IUGR biopsies had significantly higher numbers of preserved arteries and a significantly lower number of destroyed vessels compared to controls ($p < 0.05$, **Figure 2**). This was associated with a lack of both inEVT and enEVT invasion into the myometrium (**Supplemental Figure 1A** and **Tables 5**, **6**). Placental bed biopsies from IUGR and preeclamptic pregnancies showed a similar lack of enEVT invasion but surprisingly inEVT was unaffected and vascular transformation was not as compromised (**Figure 2**, **Tables 5**–**7**, and **Supplemental Figure 1A**). Scoring of decidual vessels and trophoblast invasion showed a small increase in preserved vessels and a low number of interstitial EVT in the IUGR samples as compared to healthy controls (**Table 5** and **Supplemental Figure 1B**).

Interestingly numbers of uterine arteries with an absence of mEVT were equal between controls and pure IUGR samples (**Table 7**). However, in the IUGR cases these mural EVT often displayed signs of fragmentation, nuclear condensation, blebbing, and loss of cytokeratin immunoreactivity, indicative of apoptosis (**Figure 1**, IUGR patient 2 and 3, asterisks). TUNEL

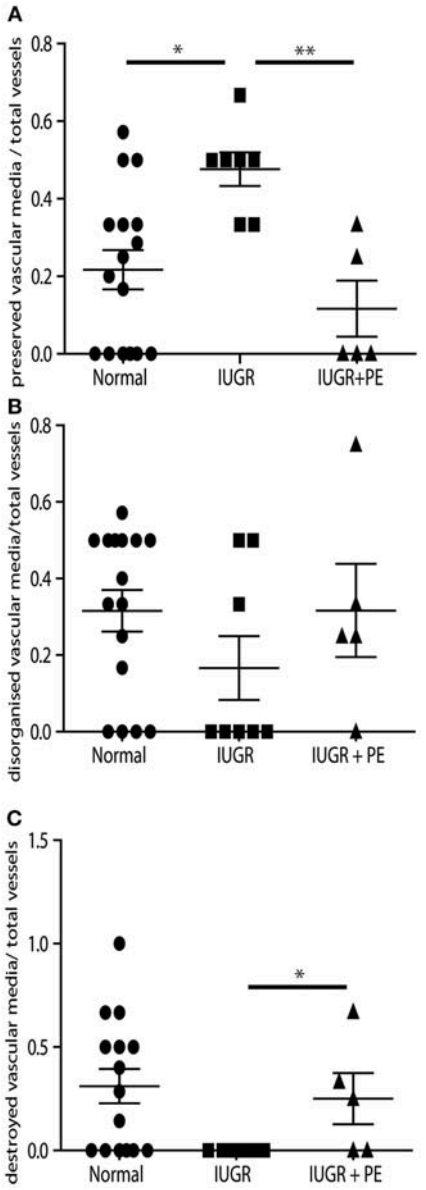

FIGURE 2 | IUGR PBBx display high numbers of preserved uterine spiral arteries. Graphs show quantitation of the degree of remodeling of all myometrial vessels control (circle), IUGR (square) and IUGR with preeclampsia (triangle) groups. Preserved vessels were scored when intact multi layered vascular smooth muscle media was present **(A)**. Disorganized vessels were scored when the vascular media was separated and smooth muscle cells were migrating away from the outer layers of the media **(B)**. Destroyed vessels were scored when all media had been lost and replaced with fibrin **(C)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

staining confirmed that these mural EVT were indeed apoptotic (arrows) showing positive staining of condensed nuclei in the IUGR cases, however the inEVT in the decidua were unaffected. Controls did not show any TUNEL reactivity in any of the EVT subtypes (**Figure 3**).

Further investigation of the maternal leukocyte populations in the PBBx was initially undertaken by immunostaining and image analysis to count the numbers of $CD45^{+}$ leukocytes present in the decidua vs. the myometrium (**Figure 4A**). Predominantly leukocytes were found in the decidua, while the few leukocytes observed in the myometrium were usually associated with the vascular plexus of the uterine vasculature (**Figure 4B**). When we compared the biopsies from the placental site (decidua basalis) with the non-placental site (decidua paretalis in the control samples we found there was no difference in the number of CD45+ leukocytes (data not shown). In contrast the numbers of CD45+ leukocytes were shown to be significantly lower in the IUGR decidua basalis as compared to control ($p < 0.001$) (**Figure 4C**). There was no significant difference between IUGR and IUGR complicated by preeclampsia. However, decidua from severe early onset preeclampsia with a normal weight baby (for gestational age) showed a significantly higher number of leukocytes in comparison to all other groups ($p < 0.0001$). In serial sections $CD45^{+}$ leukocytes were more commonly observed in dense clusters in the IUGR placental bed biopsies, and in close association with the apoptotic mural EVT (**Figure 4D**). Identification of the immune cell subtypes in these clusters

TABLE 5 | Interstitial EVT are reduced in the IUGR myometrium and decidua.

Interstitial EVT	Myometrium			Decidua	
% of vessels	Absent	Low	High	Low	High
Normal	0.00	57.14	42.86	54.55	40.91
IUGR	**89.47**	10.53	0.00	**66.67**	33.33
IUGR and PE	0.00	30.77	69.23	33.33	**66.67**

Data is presented as a percentage of all vessels in each PBBx section. Bold text indicates a significant difference from non-bold text, assessed by unpaired T-test with Welch's correction, $P < 0.05$.

TABLE 6 | Endovascular EVT are absent in IUGR and IUGR and PE myometrium.

Endovascular EVT	Myometrium		Decidua	
% of vessels	Present	Absent	Present	Absent
Normal	32.14	67.86	61.36	38.64
IUGR	15.79	**84.21**	57.14	42.86
IUGR and PE	7.69	**92.31**	50.00	50.00

Data is presented as a percentage of all vessels in each PBBx section. Bold text indicates a significant difference from non-bold text, assessed by unpaired T-test with Welch's correction, $P < 0.05$.

TABLE 7 | Intramural EVT are reduced in the IUGR decidua and myometrium.

Intramural EVT	Myometrium		Decidua	
% of vessels	Present	Absent	Present	Absent
Normal	37.50	**62.50**	**75.00**	25.00
IUGR	31.58	**68.42**	52.38	47.62
IUGR and PE	15.38	**84.62**	**83.33**	16.67

Data is presented as a percentage of all vessels in each PBBx section. Bold text indicates a significant difference from non-bold text, assessed by unpaired T-test with Welch's correction, $P < 0.05$.

showed the presence of immunoreactive $CD68^+$ macrophage, $CD3^+$ T cells and both immature $CD209^+$ and mature $CD83^+$ dendritic cells (arrows) (**Figure 4D**, IUGR Patient 1 and 2 matches to **Figure 1**). In control placental bed biopsies leukocytes were not found in association with mural EVT in the fibrin matrix of transformed vessels and mature CD83 dendritic cells were very infrequent (**Supplemental Figure 2**).

Failed Decidualization and Immune Dysregulation in the 2nd Trimester Is Associated With High Uterine Artery PI and Growth Retardation

To gain insight into the origin of the failed vascular transformation and potential immune dysregulation observed in the third trimester IUGR samples we collected decidua from women undergoing 2nd trimester termination for medical reasons. Prior to the surgical procedure uterine artery Doppler analysis was performed. Patients were grouped by uterine artery Doppler indices specific to the 2nd trimester into normal ($n = 6$), and high uterine artery PI (>1.6) with early end-diastolic notches ($n = 6$) which has been shown to be associated with an increased incidence of preeclampsia at this time point in gestation (49). Fetal abnormalities were chromosomal (T21, T18), genetic (Turners syndrome, Amniotic band syndrome, or 48XY deletion) or no fetal heartbeat. Interestingly when patient data was collated 4 of the 6 high uterine artery PI group had small for gestational age (SGA) fetuses (fetal size was assessed crown to rump length by ultrasound and was noted as expected size of 2 weeks earlier than actual gestational age). In this study we have designated this group as high uterine artery PI and small for gestational age (SGA). We do however acknowledge that we cannot exclude an effect of the fetal abnormality on the fetal size due to the small size of the patient groups; only one pregnancy was terminated at 18-weeks for pure severe IUGR. The control group had normal uterine artery indices and all fetuses were of the expected size for gestational age. Equal numbers and types of fetal abnormality were found in each group (**Table 3**).

Uterovascular remodeling and 2nd trimester decidual leukocyte populations were assessed by immunolocalization, image analysis and multi-color flow cytometry studies. In the 2nd trimester control group interstitial trophoblast staining positively for both CK7 and the EVT marker HLA-G were observed in the walls of Stage III actively remodeling decidual arterioles that had disrupted VSMC and lost their endothelial cells (**Figure 5**, top panel 18 weeks normal). Fully transformed Stage IV vessels were also observed lined with enEVT. As we have previously shown in the first trimester (34), this active vascular transformation was associated with the presence of uNK and macrophage within the vessel wall (lower panel 18 weeks normal). Significant numbers of $CD3^+$ T cells were also observed in association with the vessels.

In contrast the decidua from the high uterine artery PI SGA group was characterized by the presence of InEVT in association with the vasculature, but no enEVT were observed (**Figure 5**, lower 2 panels). In 2 SGA cases abnormal smooth muscle actin staining was seen in the DSCs and arterioles maintained their VSMC wall and intact endothelium. Most importantly, we consistently observed an increased number of small lymphatic vessels leukocytes marked by both CD31 and D240/podoplanin expression (black arrow, SGA and red arrow, IUGR). Moreover, abnormal D240/podoplanin expression lining the DSCs was also observed in the high uterine artery and SGA cases These microlymphatics between the DSC are densely packed with $CD56^+$ uNK, $CD68^+$ macrophage and $CD3^+$ T cells (**Figure 5**, 18 weeks SGA and high magnification 18 weeks IUGR). Indeed, the majority of the decidual leukocytes appear to be restricted within the lymphatic vasculature in the high PI SGA cases and were not distributed in the decidual stroma, or associated with transforming vessels, as they are in normal pregnancies. In the IUGR case trophoblast were also observed within the lymphatic vasculature (CK, arrow).

Further investigation of the decidual leukocyte subsets by flow cytometry showed that numbers of total $CD45^+$ leukocytes, $CD56^+$ uNK, and $CD68^+$ macrophage were not different between normal and high uterine artery PI SGA groups and conformed to expected ratios in the second trimester when gated within either total live cells or the $CD45^+$ leukocyte population ($CD56^+$ uNK at 50–60% and $CD68^+$ macrophage at 10–20%, **Figure 6A**). In contrast, numbers of $CD3^+$ T cells were doubled in the high uterine artery with SGA fetuses' groups as compared to the controls (13.57 vs. 7.23%, $p < 0.01$, **Figure 6B**) when gated within the $CD45^+$ population. This significant increase was not seen in the total cell comparison, probably due to an increase in numbers of small DSCs in the high uterine artery PI SGA group (observed in the image analysis of DSCs described below). The T cell subpopulations were further investigated by immunostaining serial sections of decidua with anti-CD4 and antiCD8 antibodies and quantifying T cell number using image analysis. In comparison to controls the high uterine artery PI SGA group had significantly higher numbers of cytotoxic $CD8^+$ T cells ($p < 0.001$, **Figure 6C**) and a significantly decreased CD4/CD8 ratio (1.61 ± 0.39 vs. 1.212 ± 0.35, $p = 0.0028$). Both $CD4^+$ and $CD8^+$ T cells were found in increased density in the SGA cases surrounding untransformed uterine spiral arteries (arrows, **Figure 6D**). In the controls $CD4^+$ T cells were associated with the transforming vessels but $CD8^+$ T cells were very small and infrequent **Figure 6D**, arrows).

The maturation status of decidual dendritic populations was assessed in more detail in the high granular $CD45^+$ gate by flow cytometry using CD209/DC-SIGN (immature), CD205 (intermediate), and CD83 (mature antigen presenting) antibodies. A representative comparison of 18 weeks normal and high Uterine artery PI SGA cases is shown in **Figure 7A**. As can be seen, and as we have previously shown (27), dendritic cells in the normal 2nd trimester decidua are maintained in an immature state only expressing CD209 and CD205. In contrast the high uterine artery PI IUGR case shows the appearance of a significant $CD205^+$ $CD83^+$ population that is absent in the control (45.96 vs. 3.58%). Quantification within the CD45 gate across all cases in the respective groups showed that the increase in numbers of $CD83^+$ mature dendritic cells was common to the high Uterine artery PI SGA group (**Figure 7B**, $p < 0.001$).

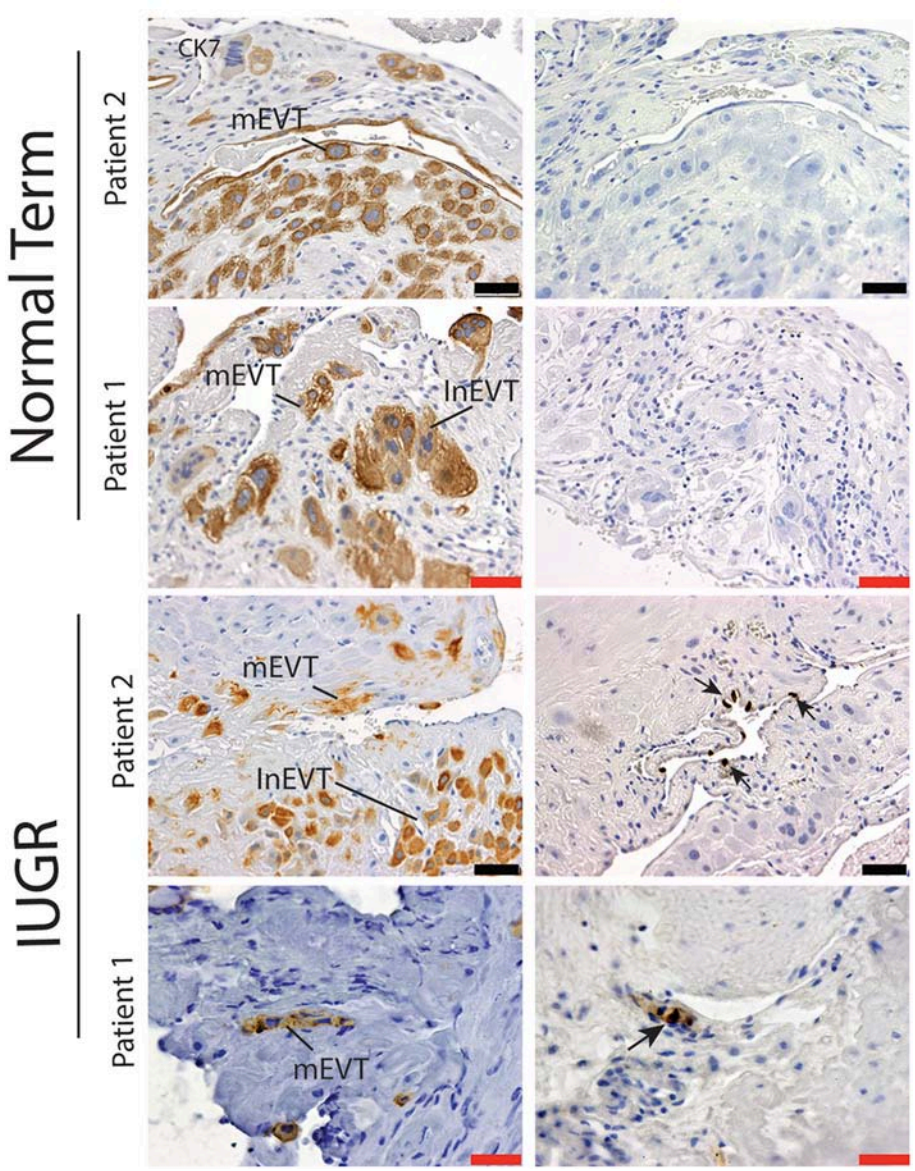

FIGURE 3 | Failed uterovascular transformation in IUGR is associated with the presence of apoptotic mural EVT. Immunohistochemical staining for cytokeratin 7 (CK7, Column 1) and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Column 2) was perfomed to confirm that the fragmented mural EVT that we observed in the IUGR cases were apoptotic. Representative serial sections of control ($n = 2/15$) and IUGR PBBx ($n = 2/8$) uterine spiral arteries at the decidual myometrial junction are shown. Arrows indicate the TUNEL positively nuclei of the mural EVT (mEVT) showing cytokeratin loss in the matching serial sections in the IUGR samples. No TUNEL staining was seen in the interstitial EVT (inEVT) of the IUGR cases or any of the EVT populations of the control term samples. Scale bars: black = 50 μM, 100x magnification; red = 20 μM, 200x.magnification.

These $CD83^+$ dendritic cells were observed in leukocyte clusters in association with unremodeled vessels and in lymphatic vessels in the high uterine artery PI SGA cases (**Figure 7C**, SGA, arrows).

Correlation of the above results supported our hypothesis that the initial failure of early decidualization may underlie the resulting uteroplacental pathology of severe IUGR. To investigate this final step in our hypothesis we investigated DSC differentiation status using 3 markers. Progesterone is known to play a key role in the decidualization of DSCs by binding to its nuclear receptor PR-B (50). IGFBP-1 is expressed by DSC as they differentiate to their secretory phenotype (51) and CD10 is expressed by mature DSC (52). To investigate if lower levels of these DSC markers correlated with the above changes in the high uterine artery PI decidua, we performed immunostaining and image analysis to count the numbers of PR-B positive nuclei in the DSC or quantify the intensity of staining in the IGFBP-1 and CD10 slides. DSC size was also quantified by ellipsoid area measurements of 3 random fields

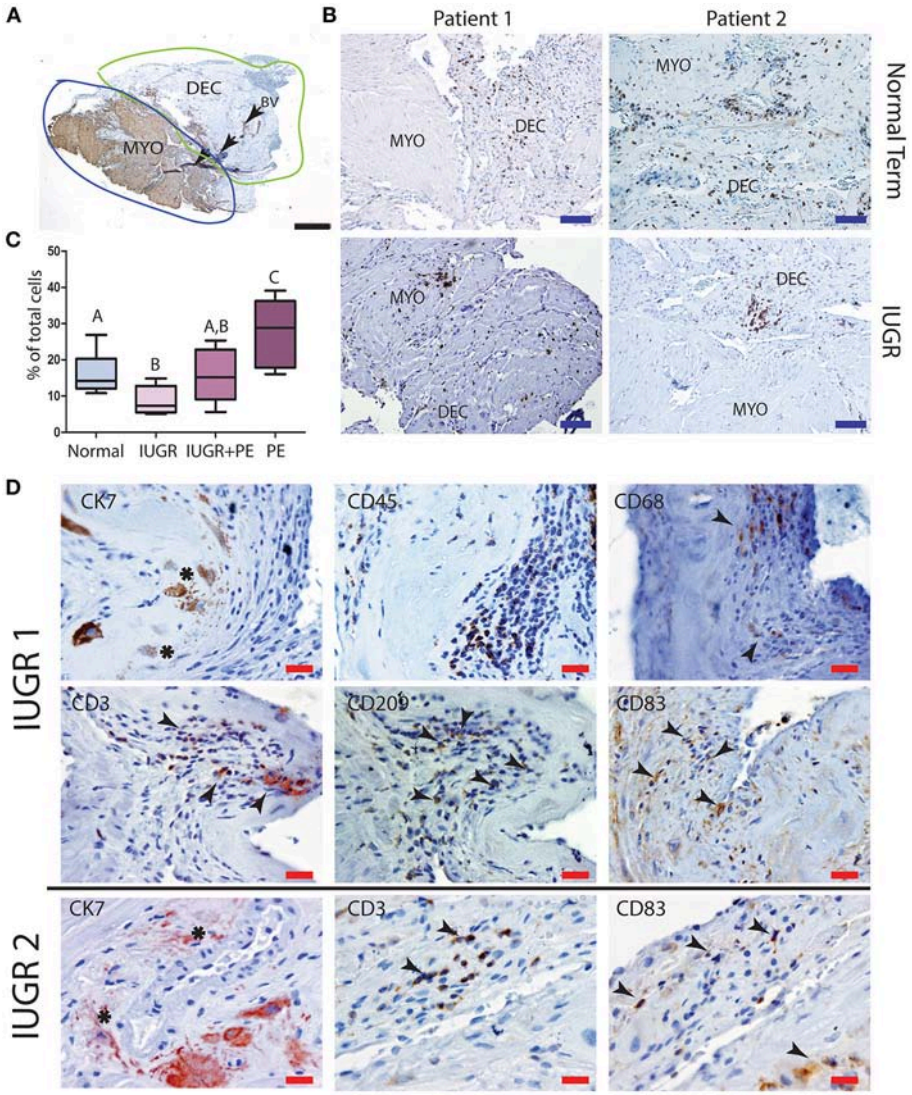

FIGURE 4 | Low decidual leukocyte number and increased mature dendritic cells in association with apoptotic EVT in IUGR. Visiopharm Newcast image analysis system was used to define decidual and myometrial regions based on smooth muscle actin staining of the myometrium **(A)**. Representative stained decidual and myometrial sections from normal term PBBx upper panel and IUGR PBBx lower panel **(B)**. Numbers of brown stained CD45+ leukocytes and blue hematoxylin stained decidual stromal cells or myometrial muscle cells were counted in corresponding serial sections across 5% of the respective region using Visiopharm Newcast image analysis system and random meander sampling **(C)**: Normal $n = 15$, IUGR $n = 8$, IUGR and PE $n = 5$, PE $n = 8$) ($^{A,B}p < 0.01$, $^{A-C}p < 0.0001$, $^{B,C}p < 0.001$). Representative serial sections of IUGR PBBx show that the apoptotic mural EVT (CK7, asterisks) that were observed in these biopsies were associated with leukocyte clusters (CD45) containing CD68+ macrophage and elevated number of CD3 T cells and CD83+ mature dendritic cells (**D**, arrows). Scale bars: black = 500 μM, 20x magnification, blue = 250 μM, 40x magnification, red = 20 μM, 200x.magnification.

of each decidual section (**Figure 8A**). Control 2nd trimester decidua was characterized by large plump pale DSC with large pale nuclei, known to be indicative of a highly active cell. In contrast, the decidual cells of the decidua were physically much smaller, stained more intensely with hematoxylin and displayed small dark staining nuclei. Area measurements showed that control DSC were much larger than decidual cells from the high uterine artery PI SGA cases (930.8 ± 116.6 vs. 670.7 ± 175.9, $p < 0.05$). In the 2 SGA cases showing the decidua also displayed elongated rectangular cells arranged in bands separated by ECM that also expressed abnormal focal points of SMA (**Figure 8D**). Many gaps were also observed between the decidual cells in the high uterine artery PI decidua corresponding to the elevated number of lymphatic vessels reported above (**Figure 8D,** arrow). In the markers of decidualization analysis more DSC expressed strong nuclear PR-B staining in the control normal samples (38.84 ± 2.57% vs. 23.56 ± 6.66%, $p < 0.0001$) (**Figure 8C**). Interestingly IGFBP-1 was restricted to areas surrounding the uterine spiral arteries in 14 and 15 weeks samples and increased to the majority of control DSC at 17–19 weeks of

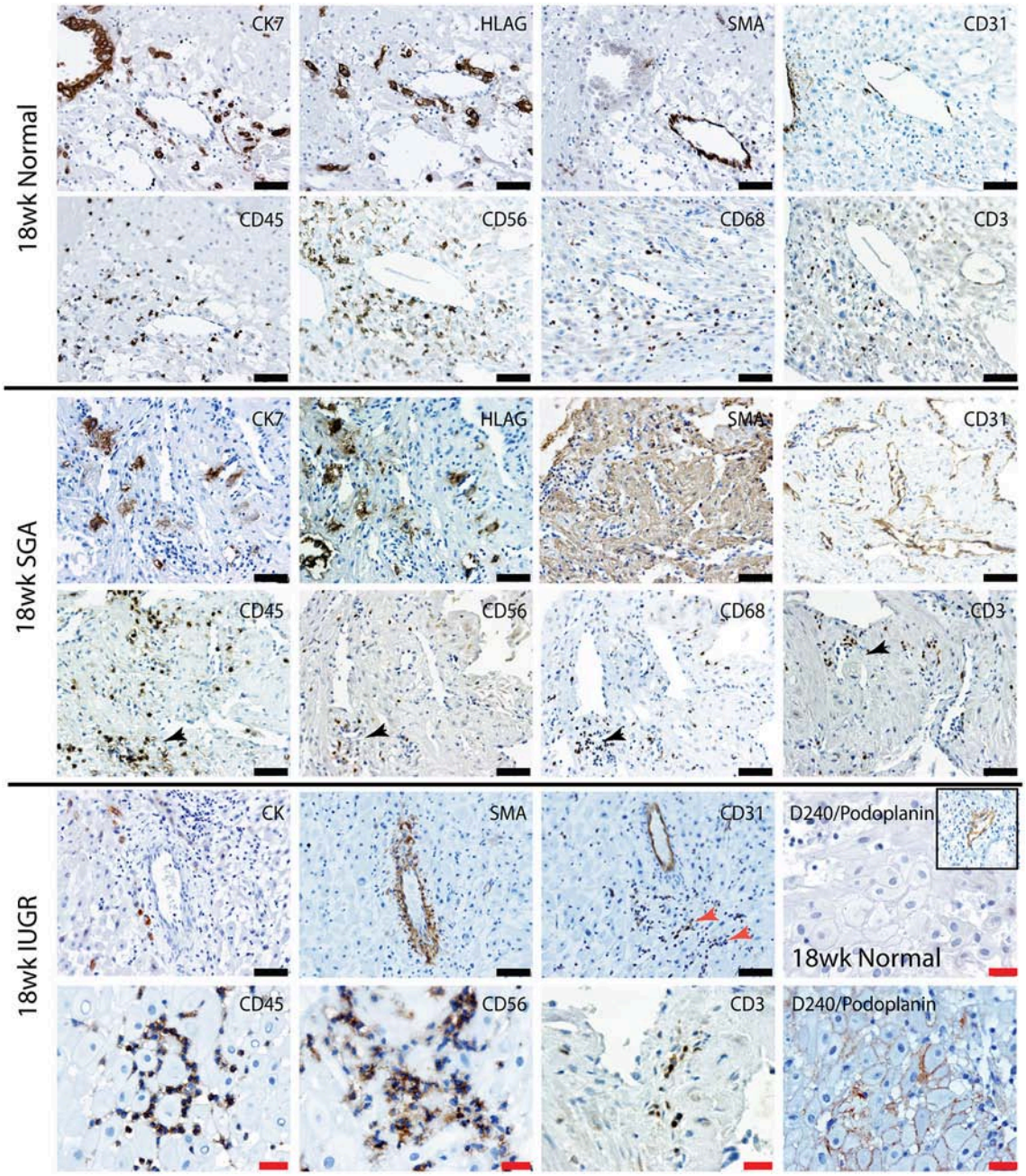

FIGURE 5 | Lymphatic vessel density is increased, and leukocytes are abnormally distributed in the high uterine artery PI SGA decidua. Representative serial sections from normal 18 week trimester decidua (top panel), an 18 week high uterine artery PI SGA case (middle panel) and a 18 week high uterine artery case of IUGR (lower panel) stained with antibodies against CK7 (trophoblast) HLAG (EVT), SMA (VSMC), CD31 (endothelial), CD45 (leukocytes) CD56 (uNK) CD68 (macrophage and CD3 (T cells). Lymphatic vessels were identified by staining with D240/podoplanin (lower right 2 images control and IUGR). In the SGA high numbers of small CD31 vessels full of leukocytes were seen (black arrows) In the IUGR decidua this was more pronounced (red arrow), abnormal D240 podoplanin lymphatic endothelial expression was found surrounding the DSC (lower right panel) creating micro-lymphatic vessels that were packed with uNK (CD56) and T cells (CD3). In controls the DSC were negative and only the larger lymphatic vessels stained positive (inset). Scale bars: black = 50 μM, 100x magnification, red= 20μM, 200x magnification.

gestation (**Figure 8C**). CD10 was similarly expressed throughout the cytoplasm of control DSC (**Figure 8D**). In contrast both IGFBP-1 and CD10 levels were significantly reduced, sporadic, or undetectable in the high uterine artery PI SGA decidua (**Figures 8C,D**).

DISCUSSION

In summary this paper has shown that PBBx from idiopathic IUGR pregnancies with abnormal uterine artery PI (>1.5) demonstrate a higher number of intact uterine spiral arteries,

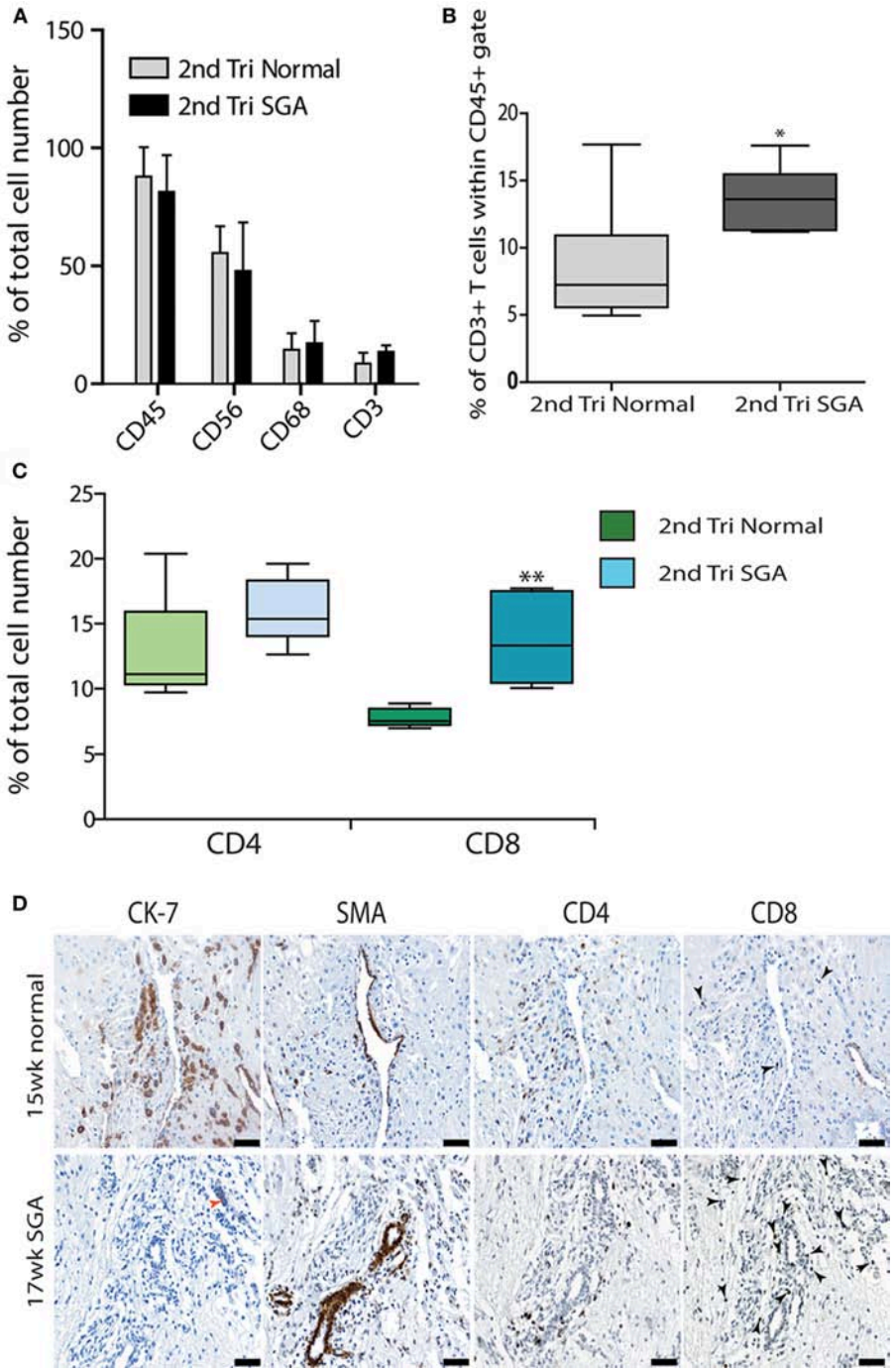

FIGURE 6 | Elevated cytotoxic $CD8^+$ T cells in 2nd trimester high Uterine artery PI and SGA decidua. Multicolored flow cytometry analysis of leukocyte subsets isolated from 2nd trimester normal or high uterine artery PI (>1.6) of decidua. Numbers of $CD45^+$ leukocytes, $CD56^{++}$ uNK, and $CD68^+$ macrophage were no different between groups **(A)**. CD3+ T cells gated within the $CD45^+$ population were doubled in the high uterine artery PI decidua **(B)**. Visiopharm Newcast image analysis system and random meander sampling was used to quantify numbers of $CD4^+$ T cells and $CD8^+$ T cells in 5% of the total tissue area of each decidual section. Percentages of the total cell number were calculated and are presented as box whisker plots in **(C)**. Representative serial sections from a 15-week normal sample (upper panel) and a 17-week high uterine artery PI SGA) stained with CK7, SMA CD4 and CD8 antibodies are shown in **(D)** (arrows identify CD8+ T cells). $^*p < 0.05$, $^{**}p < 0.01$, $n = 6$ in each group. Scale bars = 20 μM, 200x magnification.

a failure of both interstitial and endovascular invasion into the decidual myometrial junction, and high numbers of apoptotic mural and interstitial EVT. Numbers of decidual leukocytes were lower in IUGR, while mature $CD83^+$ dendritic and $CD3^+$ T cells were found in clusters in association with apoptotic EVT in the myometrial portions of these vessels. In the 2nd trimester

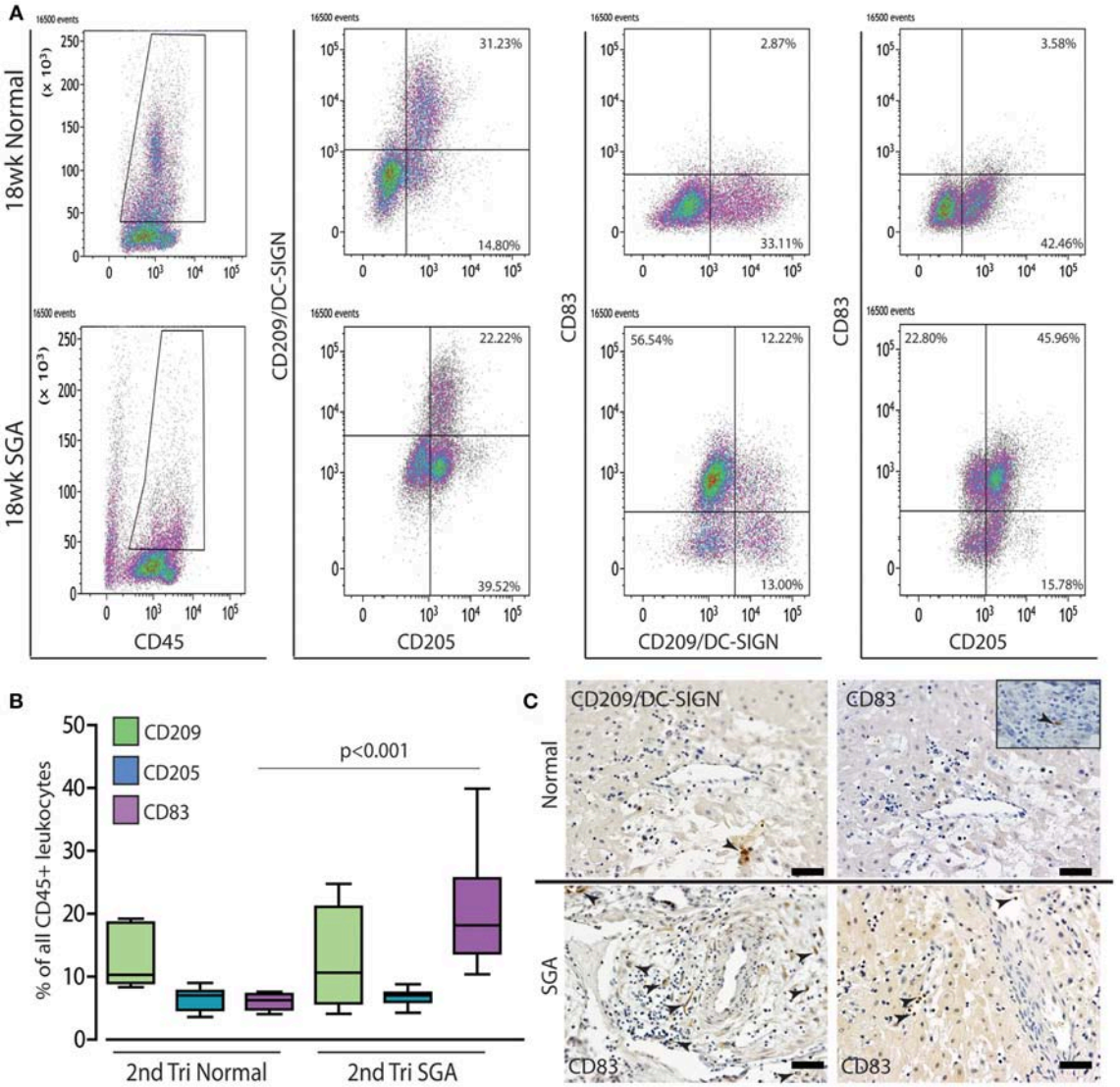

FIGURE 7 | Mature dendritic cells are significantly increased in the 2nd trimester high uterine artery SGA decidua. Representative dot plots of multicolor flow analysis of decidual dendritic cells from an 18-week normal decidua and a decidua from an 18-week high uterine artery PI SGA case. Dendritic differentiation was assessed using three markers CD209/DC-SIGN to mark immature dendritic cells, CD205 to mark intermediate dendritic cells and CD85 to mark mature antigen presenting dendritic cells. Gates were set in the high granular region of the CD45+ gate **(A)**. Comparison across all samples showed that mature CD83+ dendritic cells are increased to 20% of all $CD45^+$ leukocytes compared to 4%+- in the controls **(B)** ($p < 0.001$, $n = 6$ per group). Representative immunostained images from one control and 2 high uterine artery PI SGA cases, CD209: immature dendritic cells, CD83: mature antigen presenting dendritic cells (**C**, arrows indicate positively stained cells). Inset shows rare $CD83^+$ dendritic cell in control decidua. Scale bar = 20 μM, 200x magnification.

decidua with high uterine artery PI (>1.6) and SGA fetuses we similarly observed elevated numbers of $CD8^+$ T cells and mature $CD205^+$ $CD83^+$ dendritic cells. Most surprisingly we observed that these high uterine artery PI cases were characterized by increased numbers of lymphatic micro vessels that were packed with decidual leukocytes and a lower expression of markers of mature secretory DSC. Collectively these results provide the first direct evidence that failed progesterone mediated decidualization of the DSCs in early pregnancy leads to (1) persistence of the endometrial lymphatic vessels, (2) a failure of the DSC to recruit the leukocytes into the decidual stroma, and (3) a failure of EVT invasion and developmental maternal immune tolerance in cases of idiopathic IUGR.

Our findings regarding the lack of endovascular EVT invasion in the placental bed biopsies from idiopathic IUGR pregnancies with known abnormal uterine PI suggest that this feature is common with that of pregnancies complicated by hypertension and preeclampsia (3, 5, 15). However, there is some discrepancy regarding the complete lack of interstitial myometrial invasion that we observed in this study. Interstitial EVT were present

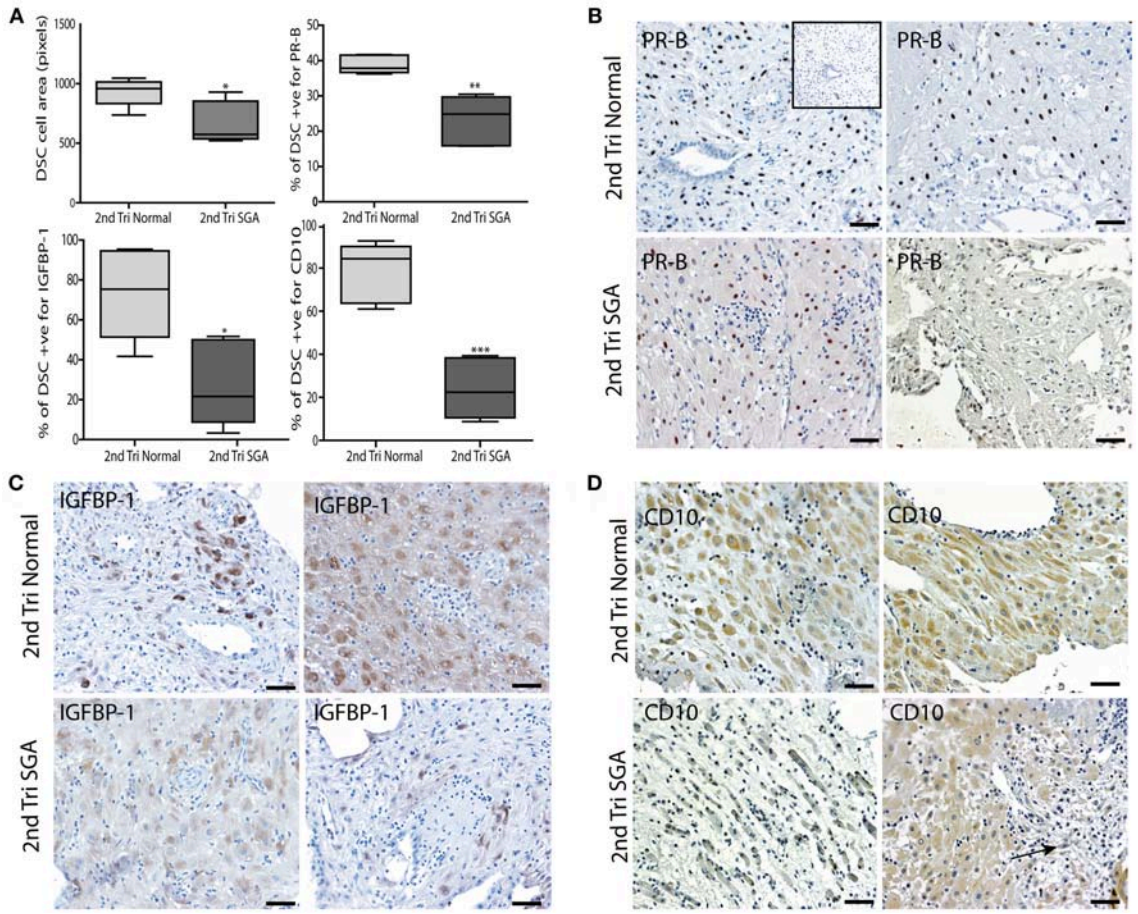

FIGURE 8 | DSC differentiation fails in the 2nd trimester high uterine artery PI SGA decidua. **(A)** DSC cell area was measured using the random ellipsoid measurement tool from the Olympus Cellsens software. Immunohistochemical image analysis was performed on serial decidual sections stained with antibodies against the decidual markers PR-B, IGFBP-1, and CD10. Visiopharm Newcast image analysis system and random meander sampling was used to quantify numbers of positively stained DSC in 5% of the total tissue area of each decidual section. Percentages of the total cell number were calculated and are presented as box whisker plots in **(A)**. Representative photographs of serial sections from 2normal 2nd trimester decidua (top panels), and 2 high uterine artery PI SGA cases (lower panels) in each case are shown stained with PR-B **(B)**, IGFBP-1**(C)** and CD10 **(D)** antibodies (arrow indicates area of increased lymphatic vessel density). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$, $n = 6$ in each group. Inset shows no staining with the Rabbit IgG negative control. Scale bar = 50 μM, 100x magnification.

at low levels in all decidual tissues associated with the IUGR biopsies but appeared to be unable to penetrate the myometrium and many accumulated and fused at the decidual myometrial junction. There is a paucity of studies on PBBx from pure idiopathic IUGR as compared to mixed cases. In a similar study to ours, Lyall et al. contrastingly reported an increase in interstitial EVT density in association with both decidual and myometrial vessels in fetal growth restriction as compared to PE and controls (14). In their study, only 60% of patients in the growth restricted groups displayed a mean uterine artery PI >95th percentile or bilateral notching, while in our study we specifically collected idiopathic IUGR cases with confirmed elevated and abnormal uterine artery PI (>1.5), this may account for the differences between the two studies. In contrast to the pure IUGR cases, the combined IUGR and preeclamptic biopsies in the current study did demonstrate high levels of interstitial invasion into the myometrium suggesting a distinct etiology of pure IUGR. Interestingly EVT from IUGR placenta have been shown to express lower levels of the VCAM-1, alpha 2 beta 1, alpha 3 beta 1, and alpha 5 beta 1 integrin's but much higher levels of intercellular adhesion molecule 3 (ICAM3) than EVT from normal pregnancies (53). The downregulation of these integrin's is likely to negatively affect the ability of the EVT to interact with collagen, laminin and fibronectin, all major extracellular matrix components of the myometrium and decidua, and thus may underlie to inability of the EVT to penetrate the myometrium. Interestingly ICAM3 binds to DCSIGN/CD209+ immature dendritic cells that are found in the decidua, suggesting that it's over expression may lead to changes in the immunological reactions between the mother and the developing fetus and placenta.

During human pregnancy the semi-allogeneic/allogeneic fetal graft is normally accepted by the mother's immune system. Initially the contact between maternal and fetal cells is restricted

to the decidua and depends on the reaction between KIR, and NKp receptors on the maternal uNK with paternally-derived non-self-human leukocyte antigens (HLA) class I and class I allotypes (54–56). In our quantitative study decidual leukocytes were shown to be lower in IUGR pregnancies but higher in preeclampsia. We suggest that this is likely to be reflective of a reported decrease in the numbers of uNK in the term IUGR decidua (41). Correlation of this data with the observed increase in the numbers of preserved or partially remodeled decidual vessels, and decreased numbers of enEVT (**Supplemental Figure 1B**) suggests that this critical interaction of EVT and uNK in early tolerance and decidual vascular transformation is compromised in IUGR.

In the myometrium maternal allo-recognition of the fetus depends on avoidance of maternal T cells recognizing and responding to fetal antigen, due to the restriction of the $CD56^+$ uNK to the decidual compartment (57). It is also known that in response to trophoblast-secreted factors, decidual $CD209^+$ dendritic cells induce differentiation of $CD4^+CD25^-$ T cells into a $CD4^+CD25^+FOXP3^+$ Treg population capable of inhibiting $CD4^+$ T lymphocyte proliferation (58). In our study we observed apoptotic TUNEL positive mural EVT near maternal leukocyte clusters of macrophage, mature $CD83^+$ antigen presenting dendritic cells and $CD3^+$ T cells. Recently depletion or malfunction of T cell populations has been associated with recurrent spontaneous abortion and incidence of preeclampsia (59, 60) and higher numbers of $CD209^+$ and $CD83^+$ DC have been reported in the preeclamptic placental bed (61). Correlation of these studies suggests that the maternal response against the trophoblast may be hostile and excessive in all of these placental pathologies. This is further supported by our quantification of decidual leukocyte populations in the second trimester with high uterine artery PI and SGA fetuses where we found a significant increase in the numbers of cytotoxic $CD8^+$ T cells and mature $CD83^+$ dendritic cells as compared to the control group. Mature CD83+ dendritic cells are capable of presenting fetal antigen to the adaptive immune cells (62). We have previously shown that in healthy 2nd trimester pregnancy the proportion of $CD3^+$ T cells doubles from 1st to 2nd second trimester from 10 to 20% of all $CD45^+$ leukocytes in the decidua and is predominated by Th2 $CD4^+$ and Th1 $CD8^+$ populations, while decidual dendritic cells are maintained in an immature status (27). In the current study the control samples similarly displayed a normal CD4:CD8 ratio (i.e., close to 2 while the high uterine artery PI SGA cases displayed a ratio of 1.21 due to the elevation in CD8+ T cells; this change in T cell ratio is indicative of altered immune function, and chronic inflammation (63). Thus, we suggest that the results in the current study support the hypothesis of activation of the maternal immune system against the fetus and placenta in these cases of maternal idiopathic IUGR. Elevation of CD8 T cells has also previously been reported in the maternal peripheral circulation in cases of IUGR (64). Furthermore, these findings suggest a contributory mechanism to the increased incidence of villitis of unknown origin (VUE), an inflammatory condition of the placenta characterized by maternal T cell infiltrates in the villous stroma in fetal growth restriction and stillbirth (65, 66). In our patient group two of eight IUGR placentas displayed signs of VUE.

The second trimester decidua with high uterine artery PI also provided an unexpected insight into the potential global decidual contribution to the utero-placental pathology of IUGR. It should however be noted that although 4 of 6 cases were shown to be 2 weeks smaller than expected for their gestational age (thus designated as SGA) we cannot conclusively say that these pregnancies, if healthy, would have progressed to IUGR rather than preeclampsia as elevated uterine artery PI has been shown to be predictive for the onset of preeclampsia (49). The most surprising finding in these cases was the persistence of significant numbers of micro-lymphatic podoplanin positive vessels in association with the decidual spiral arterioles. In the basal layer of the non-pregnant endometrium lymphatic vessels are reported to be closely associated with the spiral arterioles (67) and to regress with the onset of perivascular decidualization of the DSC in the late secretory phase such that they are rarely observed in the decidua (68–71). Higher numbers of lymphatic vessels have also been recently found in the decidua of recurrent spontaneous abortions (72), suggestive of a common defect in decidualization in these conditions. In the high uterine artery PI and SGA cases the lymphatic vessels were also packed with decidual leukocytes of uNK, macrophage and T cell lineages, while very few leukocytes were seen in the decidual stroma as is normally observed. Differentiated DSC cells secrete numerous chemokines critical to the recruitment and trafficking of these leukocyte subsets out of the lymphatics and into the stroma, where they play key role in early vascular transformation (21). This again suggested that the decidualization of the DSC was compromised in the high uterine artery PI and SGA cases. Of note, in addition to invading and transforming the uterine arteries EVT have also recently been shown to invade the walls and endothelium of veins and glands and lymphatic vessels in the decidua basalis (72–74). We also observed EVT in venous and lymphatic vessels in both control and high uterine artery PI and SGA cases (**Figure 5**).

Progesterone is known to play a key role in decidualization of the stromal cells and its effects are primarily mediated via its PR-B receptor. A recent study has shown that in human endometrial stromal cells, PR-B regulates a significantly larger transcriptome than PR-A occupying unique binding sites associated with suppression of cell cycle regulators and induction of angiogenic growth factors and chemokine expression known to be critical in DSC interactions with trophoblast and immune cells (50). In this study we have shown for the first time progesterone responsiveness is decreased in 2nd trimester high uterine artery PI SGA decidua as compared to normal PI controls. The stromal cells of these cases were much smaller and displayed abnormal cellular morphology and far fewer expressed nuclear PR-B and lower levels of the DSC markers IGFBP-1 and CD10. Moreover, PR-B and IGFBP-1 was absent in the most abnormal growth restricted cases expressing SMA in the decidua. The mechanisms underlying PR-B dysregulation are not known however defective menstrual conditioning or epigenetic modulation of PR-B expression has been proposed (75, 76). Interestingly both estrogen and progesterone levels have recently

been shown to be lower in the serum of IUGR pregnancies than healthy controls (77), and a recent mouse model of stress induced IUGR has also shown that pregnant dams exhibited reduced progesterone levels and placental heme oxygenase 1 (Hmox1) expression and an increase in cytotoxic CD8+ T cells (78). We therefore conclude, in accordance with the hypothesis proposed by Pijnenborg et al. (30), that progesterone mediated decidualization is negatively affected in cases of high Uterine artery PI and SGA. Interestingly, 7 out of 8 patients in our IUGR PBBx group had either recurrent IUGR or high gravidity but low parity ($G > 3$, $P < 1$) suggestive of a recurrent problem with decidualization, implantation or maintenance of pregnancy.

In conclusion, we have shown that failure of the first step of progesterone mediated DSC decidualization has a fundamental and detrimental effect on all subsequent stages of decidual development in the second trimester. The regression of the decidual lymphatics, migration of the immune cells into the decidual stroma, maternal adaptive immune tolerance, EVT invasion and trophoblast-leukocyte mediated uterovascular transformation were all compromised. Correlation of these findings with our observations in placental bed biopsies supports the hypothesis that defective decidualization leads to the uteroplacental pathology observed in cases of IUGR.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CD and SL conceptualized the research program and secured the grant funding. CD, CM, SK, JK, and SL conceived and designed the placental bed biopsy study. CD, LH, RJ, and WW conceived and designed the second trimester study. CD, JW, AH, MK, and SW performed the experiments and analyzed the data. CM, WW, and JK recruited and consented patients. CD and CM wrote the manuscript. LH, CD, AH, and CM revised the manuscript. All authors read the manuscript and approved its submission.

FUNDING

The study was supported by grants to CD and SL from the Canadian Institutes of Health Research CIHR MOP-86322, CIHR MOP-82811, and FDN-143262. JW, MK, and AH were all recipients of the Richard Venn and Carol Mitchell Graduate Studentship in Women's Health Research at the Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital. CM work was supported in part by the Whitman Fellowship from Harvard Medical School, Boston, MA.

ACKNOWLEDGMENTS

The authors thank the donors, and the Research Center for Women's and Infants' Health BioBank Program of the CIHR Group in Development and Fetal Health (CIHR MGC-13299), the Lunenfeld Tanenbaum Research Institute, and the MSH/UHN Dept. of Obstetrics & Gynecology for the human specimens used in this study. We thank Ms. Annie Bang of the Flow Cytometry Facility (LTRI, Mount Sinai Hospital) for the technical support with flow cytometry and Milan Ganguly from the pathology department for performing the TUNEL staining assay.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00160/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 21 March 2019
doi: 10.3389/fendo.2019.00165

Human sFLT1 Leads to Severe Changes in Placental Differentiation and Vascularization in a Transgenic hsFLT1/rtTA FGR Mouse Model

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Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *19 October 2018*
Accepted: *27 February 2019*
Published: *21 March 2019*

Citation:
Vogtmann R, Kühnel E, Dicke N, Verkaik-Schakel RN, Plösch T, Schorle H, Stojanovska V, Herse F, Köninger A, Kimmig R, Winterhager E and Gellhaus A (2019) Human sFLT1 Leads to Severe Changes in Placental Differentiation and Vascularization in a Transgenic hsFLT1/rtTA FGR Mouse Model. Front. Endocrinol. 10:165. doi: 10.3389/fendo.2019.00165

The anti-angiogenic soluble fms-like tyrosine kinase 1 (sFLT1) is one of the candidates in the progression of preeclampsia, often associated with fetal growth restriction (FGR). Therapeutic agents against preeclampsia with/without FGR, as well as adequate transgenic sFLT1 mouse models for testing such agents, are still missing. Much is known about sFLT1–mediated endothelial dysfunction in several tissues; however, the influence of sFLT1 on placental and fetal development is currently unknown. We hypothesize that sFLT1 is involved in the progression of FGR by influencing placental differentiation and vascularization and is a prime candidate for interventional strategies. Therefore, we generated transgenic inducible human sFLT1/reverse tetracycline-controlled transactivator (hsFLT1/rtTA) mice, in which hsFLT1 is ubiquitously overexpressed during pregnancy in dams and according to the genetics in hsFLT1/rtTA homozygous and heterozygous fetuses. Induction of hsFLT1 led to elevated hsFLT1 levels in the serum of dams and on mRNA level in all placentas and hetero-/homozygous fetuses, resulting in FGR in all fetuses at term. The strongest effects in respect to FGR were observed in the hsFLT1/rtTA homozygous fetuses, which exhibited the highest hsFLT1 levels. Only fetal hsFLT1 expression led to impaired placental morphology characterized by reduced placental efficiency, enlarged maternal sinusoids, reduced fetal capillaries, and impaired labyrinthine differentiation, associated with increased apoptosis. Besides impaired placental vascularization, the expression of several transporter systems, such as glucose transporter 1 and 3 (*Glut-1*; *Glut-3*); amino acid transporters, solute carrier family 38, member one and two (*Slc38a1*; *Slc38a2*); and most severely the fatty acid translocase *Cd36* and fatty acid binding protein 3 (*Fabp3*) was reduced upon hsFLT1 expression, associated with an accumulation of phospholipids in the maternal serum. Moreover, the Vegf pathway showed alterations, resulting in reduced Vegf, Vegfb, and Plgf protein levels and increased Bad and Caspase 9 mRNA levels. We suggest that hsFLT1 exerts an inhibitory influence on placental vascularization

by reducing Vegf signaling, which leads to apoptosis in fetal vessels, impairing placental differentiation, and the nutrient exchange function of the labyrinth. These effects were more pronounced when both the dam and the fetus expressed hsFLT1 and ultimately result in FGR and resemble the preeclamptic phenotype in humans.

Keywords: human sFLT1, fetal growth restriction, vascularization, placenta, transgenic mouse model

INTRODUCTION

Appropriate development and growth of the fetus depend on adequate vascularization of both fetus and mother at the feto-maternal unit; this vascularization involves uterine vasodilation and vessel remodeling upon trophoblast invasion, as well as vasculo- and angiogenesis within the placenta. The consequences of abnormal vascular development have been associated with various pregnancy-related pathologies, ranging from miscarriage to fetal growth restriction (FGR) or preeclampsia (PE) (1).

At least 60% of the 4 million neonatal deaths that occur worldwide each year are associated with low birth weight caused by FGR, which is characterized by insufficient growth during pregnancy or preterm delivery (2). Epidemiologic studies have found that children born with FGR are a risk cohort with increased incidence and prevalence of diseases such as short- and long-term metabolic and cardiovascular alterations, adiposity, and neurological disorders in later life (3–5). Currently there are no treatments for FGR.

FGR is one of the main consequences of the pregnancy disorder PE, which is characterized by maternal hypertension and proteinuria. PE is still a leading cause of maternal and neonatal mortality (6, 7) and is often associated, amongst other factors such as oxidative stress and genetic factors, with an overexpression of the angiogenesis inhibitor soluble fms-like tyrosine kinase 1 (sFLT1) in the placenta as the pregnancy progresses (7, 8). sFLT1 is a soluble splice variant of the membranous vascular endothelial growth factor receptor 1 (VEGFR-1), which contains only the extracellular domain. Therefore, sFLT1 acts as a decoy receptor by binding and reducing free circulating levels of the angiogenesis-promoting VEGF and placental growth factor (PlGF), thereby limiting their bioavailability. There is strong evidence that overproduction of sFLT1 in the placenta and the resulting high levels of sFLT1 in maternal serum are an important cause of vascular dysfunction associated with PE through sFLT1–dependent antagonism of VEGF (8, 9). The linkage of sFLT1 with the pathophysiology of PE was clearly shown by a study of Levine et al. (10), which found that an increase in circulating levels of sFLT1 is associated with the severity of PE. Currently the sFLT1/PlGF ratio is used as a clinical biomarker for predicting PE (11–13). In addition, an elevated level of sFLT1 leads to several maternal consequences, such as endothelial dysfunction that causes hypertension and "glomerular endotheliosis" that finally leads to cellular injury and disruption of the filtration apparatus, with proteinuria, and edema as consequences (8).

We recently confirmed the importance of sFLT1 in human pregnancy and as a clinical marker for early and late-onset PE, as well as FGR (14). This role has been further confirmed by the studies of Thadhani et al. (15, 16), showing that therapeutic apheresis, which reduces the circulating levels of sFLT1 and the severity of proteinuria in women with exceedingly preterm PE, appears to prolong pregnancy without severe adverse consequences to the mother or the fetus. Much is known about the molecular mechanisms of sFLT1, which disturbs endothelial cell function [reviewed by Lecarpentier and Tsatsaris (17)]; however, the way in which sFLT1 affects the placenta and fetus, resulting in FGR, is currently unknown. In own previous studies, using a lentiviral placenta-specific sFLT1 mouse model for FGR and PE [established by Kumasawa et al. (18)], we observed a reduction in fetal and placental weight associated with a reduction in the transporting trophoblast layer and by changes in the expression of labyrinthine nutrient transporters [(19); reviewed in Winterhager and Gellhaus (20)]. These findings demonstrate that sFLT1 not only acts directly on endothelial cells and changes endothelial physiology but also directly or indirectly affects placental development and function.

Studies using several animal models that overexpress human sFLT1 (hsFLT1) have shown that hsFLT1 causes symptoms of PE, such as hypertension and proteinuria (8). Since most of the existing PE mouse models were developed by injection of replication-deficient sFLT1 lenti- or adenoviruses, we developed doxycycline (Dox)-inducible transgenic hsFLT1 mice [hsFLT1/reverse tetracycline-controlled transactivator (rtTA) mice] to receive a stable and reproducible hsFLT1 expression. In this study we found ubiquitous maternal and placental/fetal expression of hsFLT1 or ubiquitous maternal expression only upon treatment with Dox (Tet-On System induced during midgestation). Thus, we can discriminate between the consequences of maternally expressed hsFLT1 and those of maternally and placentally/fetally expressed hsFLT1 on placental and fetal outcome. We hypothesize that elevated levels of hsFLT1, if increased in all three compartments (dam, placenta, and fetus) may influence placental function and transport capability via altered placental angiogenesis and altered Vegf signaling caused by reduced levels of growth factors such as Vegf and Plgf, thereby resulting in FGR.

MATERIALS AND METHODS

Generation of hsFLT1/rtTA Mice and Experimental Procedures

A mouse strain harboring a tetracycline-inducible cassette expressing hsFLT1 was generated according to previously published protocols (21, 22). For details of the generation of the hsFLT1/rtTA mouse and the experimental set-up, see **Figure 1A**. The mice were generated on a 129/Sv background.

The full-length *hsFLT1* cDNA (2100 bp) was inserted into pBS31_tetO_promoter/simian virus 40 5′ of the tetO minimal promoter of cytomegalovirus (CMV). KH2 embryonic stem cells (ESCs) carrying the *rtTA* transgene in the ROSA26 locus were electroporated with 50 μg of pBS31_*hsFLT1* and 25 μg of an expression vector for Flp recombinase [pCAGGS-*flpE;* (23)]. The KH2 ESCs originated from the v6.5 mouse ESC line, which was established from cells derived from the inner cell mass (ICM) of a 3.5-day-old mouse embryo from a C57BL/6 × 129/sv cross. Flp-mediated recombination of pBS31_*hsFLT1* leads to the integration of *hsFLT1* cDNA into the ColA1 locus of KH2 ESCs. This recombination initiates the expression of the promoter- and ATG-less hygromycin resistance cassette present in the Col1A1 locus. One day after electroporation, 140 μg/ml hygromycin was added; colonies were selected after 10 days; and clones were screened by Southern blotting using SpeI to digest genomic DNA and a 3′ internal probe (21). Five positive clones in which hsFLT1 expression was induced after the addition of 0.5 μg/ml Dox for 48 h were expanded. The resulting *hsFLT1*–KH2 ESCs were treated with Dox (1 mg/ml), and the cell culture supernatants were analyzed for hsFLT1 secretion with enzyme-linked immunosorbent assay [ELISA; human sVEGFR-1 Quantikine ELISA (SVR100B), R&D Systems, Minneapolis, MN, USA].

Subsequently, *hsFLT1*–KH2 ESCs were injected into blastocysts isolated from 129/Sv Jae female mice; the blastocysts were then transplanted into pseudopregnant mice. We obtained 20 chimeric mice and achieved germline transmission. The newly generated mice were registered with the mouse genome informatics database; the allele was named Col1A1$^{tm2(tetO-Flt1^*)Hsc}$ (MGI: 6202353). These mice were mated with Rosa26–*rtTA-M2* mice (B6.Gt(ROSA)26Sor$^{tm1(rtTA^*M2)Jae}$, Stock No. 006965; Jackson Laboratory, Bar Habor, ME, USA) to generate hsFLT1/rtTA double transgenic mice. The Rosa26 gene locus is ubiquitously expressed; the reverse tetracycline-controlled transactivator protein (rtTA-M2) therefore reveals nearly ubiquitous target gene expression in many tissues. According to the Jackson Laboratory, expression can be observed in liver, bone marrow, stomach, intestine, and skin, with lower levels in the heart, lungs, kidney, spleen, and thymus and no expression in the brain and testes (https://www.jax.org/strain/006965).

In the parental generation (P generation) of double transgenic hsFLT1/rtTA mice, ubiquitously expressed rtTA induces hsFLT1 expression upon treatment with Dox. When Dox is added, rtTA can bind to the TetO promoter of the *hsFLT1* transgene, leading to hsFLT1 expression (hsFLT1/rtTA+Dox/FGR group); without Dox, hsFLT1 is not expressed (hsFLT1/rtTA-Dox/control group). Single transgenic hsFLT1 mice treated with Dox do not express hsFLT1 but can be used as a control for the effects of Dox (hsFLT1+Dox/Dox control group). Since Dox passes the placental barrier, hsFLT1/rtTA homozygous (hom) and heterozygous (het) fetuses/placentas in the first filial generation (F1 generation) of the FGR group can also express hsFLT1, whereas wild-type (wt) fetuses/placentas cannot, because they do not express rtTA protein and thus the Tet-On system does not function (**Figure 1A**). Therefore, the maternal FGR group must be subdivided by the fetal *rtTA* genotype for placental and fetal analysis (**Figure 1B**). This process leads to the experimental groups shown in **Table 1**.

For experimental procedures, animals were mated overnight. When a vaginal plug was present, the following day was counted as gestational day 0.5 (day post coitum; dpc). Beginning at early to midgestation (7.5 or 10.5 dpc) until the end of pregnancy (18.5 dpc), animals were treated with either Dox and sucrose or sucrose only (**Figure 1C**). Therefore, the FGR experimental group and the Dox control group received 2 mg/ml Dox [0.2% (w/v); Merck, Darmstadt, Germany] and 30 mg/ml sucrose [3% (w/v); Carl Roth, Karlsruhe, Germany] in the drinking water, whereas the control group received only 30 mg/ml sucrose in the drinking water. The drinking water was renewed every third day. The endpoint of the experiments was set at 18.5 dpc. Mice from each experimental group were sacrificed; whole blood was collected by cardiopuncture; and fetuses, placentas, and maternal organs such as liver and kidneys were isolated and weighed. Fetal maturation, including FGR characteristics, was assessed on the basis of morphological criteria according to the Theiler developmental atlas: (http://www.emouseatlas.org/emap/ema/theiler_stages/house_mouse/book.html). The fetal to placental weight ratio was determined because it reflects the efficiency of the placenta in meeting the nutritional demands of the growing fetus (24).

All mice were housed in the animal facility of the University Hospital Essen in a specific pathogen–free environment and were exposed to cycles of 12 h of light and 12 h of dark. They were provided with food and water *ad libitum*. All animal procedures were performed in accordance with the German laws for animal protection (No.: G1265/12 and G1644/17).

Tissue Preparation

At 18.5 dpc, anesthetized pregnant mice were killed by cervical dislocation. Maternal blood was collected; maternal liver, and kidney, as well as fetuses and placentas, were dissected in sterile phosphate-buffered saline (PBS); and the amniotic membrane was removed from the placenta. Isolated fetuses and placentas were weighed with an ALJ 220-4 NM analytical balance (Kern, Ebingen, Germany) with a linearity of ±0.2 mg. Organs were either frozen and stored at −80°C (for RNA, DNA, and protein analysis) or fixed in 4% paraformaldehyde (PFA) for 24 h and stored in 70% ethanol until being embedded in paraffin standard procedures (for morphology).

Genomic DNA Isolation, Genotyping, and Sex Determination

Genomic DNA was isolated from ear punch tissue samples with the REDExtract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The quality and quantity of DNA were verified with μCuvette G1.0 and BioPhotometer Plus (Eppendorf, Hamburg, Germany). Genotyping and sex determination of mice were performed with a standard PCR program (hsFLT1: initial denaturation 95°C, 5 min; 40 cycles 94°C, 45 s, 60°C, 45 s, 72°C, 1 min, final extension 72°C, 5 min; rtTA: initial denaturation 94°C, 3 min; 35

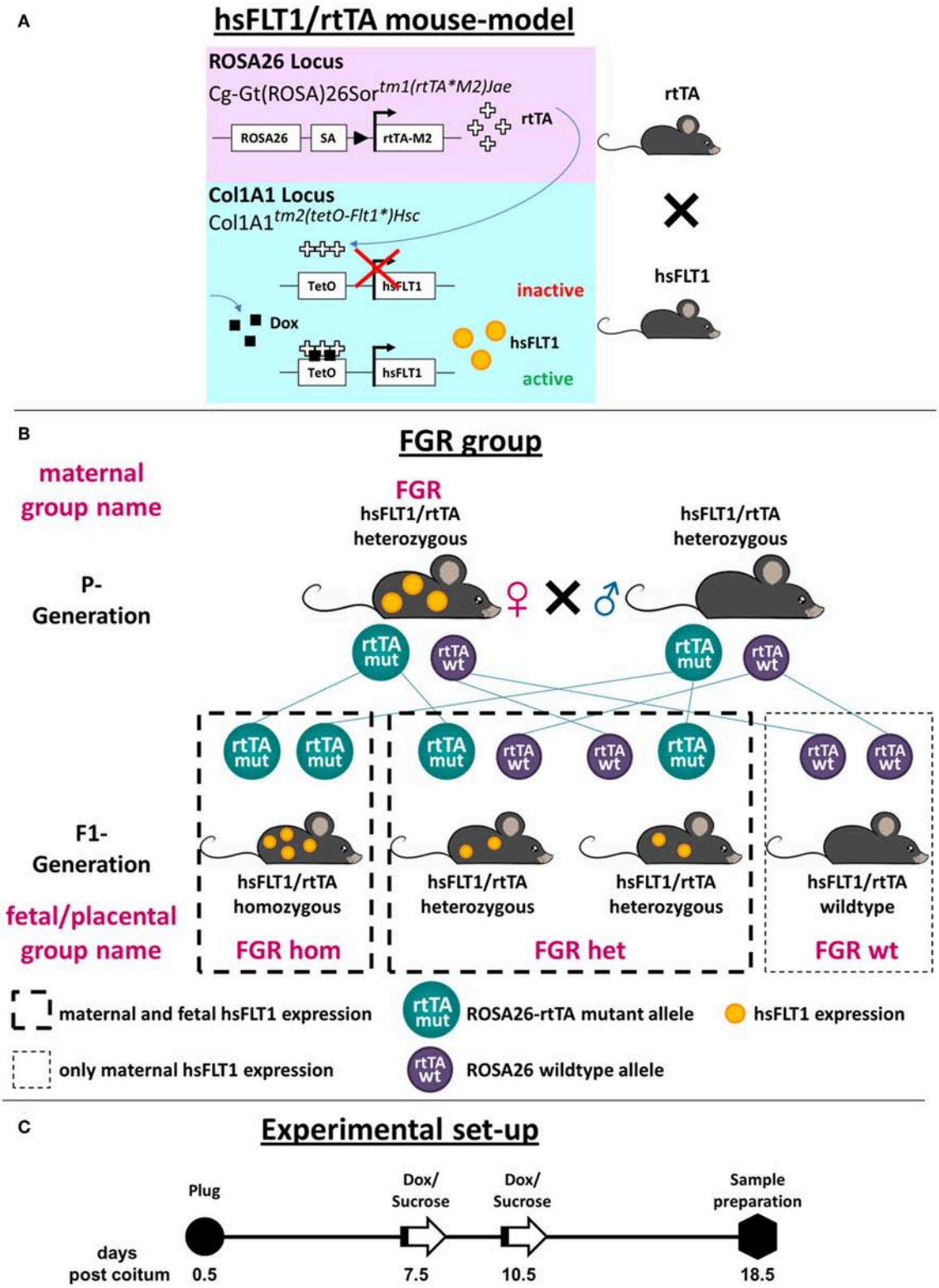

FIGURE 1 | Generation of the hsFLT1/rtTA mouse model with ubiquitous overexpression of hsFLT1. **(A)** We mated single transgenic Gt(ROSA)26Sor$^{tm1(rtTA^{*}M2)Jae}$ mice with single transgenic Col1a1$^{tm2(tetO-Flt1^{*})Hsc}$ mice to generate double transgenic human soluble fms-like tyrosine kinase 1 reverse tetracycline-controlled transactivator (hsFLT1/rtTA) mice. These mice ubiquitously express rtTA, which induces hsFLT1 expression upon treatment with doxycycline (Dox):

(Continued)

FIGURE 1 | hsFLT1/rtTA+Dox/FGR (fetal growth restriction) group. Without Dox, no expression of hsFLT1 occurred (hsFLT1/rtTA-Dox/control). Single transgenic hsFLT1 mice treated with Dox (hsFLT1+Dox/Dox control) cannot express hsFLT1 but were used as a control for Dox effects (scheme adapted from that of Hubert Schorle, Bonn). **(B)** Mating scheme of FGR group. Heterozygous sFlt-1/rtTA mice were mated. Since Dox passes the placental barrier, hsFLT1/rtTA homozygous (hom), and heterozygous (het) fetuses expressed hsFLT1, whereas wild-type (wt) fetuses did not. For this reason the FGR experimental group was subdivided into FGR hom, FGR het, and FGR wt (depending on the *rtTA* genotype) for fetal and placental analysis. P, parental; F1, first filial generation of fetuses. **(C)** Experimental set-up. Mice were mated overnight. The day after the development of a vaginal plug was defined as day 0.5 post coitum (dpc). At early to midgestation (7.5 or 10.5 dpc), dams were treated either with 2 mg Dox and 3% [w/v] sucrose per ml of drinking water or with 3% [w/v] sucrose only as a control until cesarean section and sample preparation were performed at 18.5 dpc (cesarean section and sample preparation).

TABLE 1 | Maternal and fetal experimental groups.

Maternal groups	Fetal groups
FGR (hsFLT1 expression)	FGR hom (maternal and fetal hsFLT1 expression)
	FGR het (maternal and fetal hsFLT1 expression)
	FGR wt (only maternal hsFLT1 expression)
Control (no hsFLT1 expression)	Control (no hsFLT1 expression)
Dox control (no hsFLT1 expression)	Dox control (no hsFLT1 expression)

Dox, doxycycline; FGR, fetal growth restriction; hsFLT1, human soluble fms-like tyrosine kinase 1.

cycles 94°C, 45 s, 65°C, 1 min., 72°C, 1 min, final extension 72°C, 2 min) and the appropriate primers (**Table 2**).

Genomic DNA Isolation and Pyrosequencing

Approximately 20 mg of placental tissue was homogenized with a Tissue Lyser LT (Qiagen, Hilden, Germany). Genomic DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen) according to the manufacturer's protocol. The quality and quantity of DNA were verified with Nanodrop 2000c (Thermo Fisher Scientific, Pittsburgh, PA, USA). Bisulfite conversion of 500 ng genomic DNA was performed with the EZ DNA methylation gold kit (Zymo Research, Leiden, The Netherlands) according to the manufacturer's protocol. Pyrosequencing was performed as previously described by Freitag et al. (25). The sequences of bisulfite-specific primers for long interspersed element 1 (*LINE1*), insulin-like growth factor two (Igf2) differentially methylated region two (*Igf2-DMR2*), and H19 imprinting control region (*H19-ICR*) have been previously published (25). The PCR product was analyzed for the extent of methylation per selected CpG position with a Pyromark Q48 sequencer (Qiagen). Data were analyzed with PyroMark Q48 autoprep software (Qiagen). The level of DNA methylation was given as a percentage. Samples were obtained from complete placentas. The experimental groups FGR hom ($n = 4$), FGR het ($n = 7$), and FGR wt ($n = 7$) were analyzed, as well as the control group ($n = 10$) and the Dox control group ($n = 6$).

RNA Extraction, cDNA Synthesis, and Quantitative PCR

Total RNA was extracted from ∼10 mg frozen samples of placenta, liver, kidney, and fetus with the E.Z.N.A Total RNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized with 1 μg RNA as previously described (19). Gene expression was measured from 1 μl cDNA with 19 μl of the VeriQuest® Fast SYBR® Green qPCR Mix (Affymetrix, Santa Clara, CA, USA) and the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with a standard PCR program (**Table 2**). For quantitative measurement, standard curves of 1 μl cDNA of standards with known concentrations from 1,000 to 0.1 fg of each measured gene were used. The quantitative PCR (qPCR) analyses were carried out in triplicate. The amount of cDNA in each sample was normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a housekeeping gene and experimental groups were normalized to control group. Primer sequences are listed in **Table 2**. We tested the following experimental groups: FGR hom $n = 7$, FGR het $n = 15$, control $n = 12$, Dox control $n = 13$.

Analysis of hsFLT1 Serum Levels

Blood was collected from anesthetized pregnant mice by cardiopuncture after cervical dislocation. Serum samples were prepared by centrifuging clotted blood for 15 min at 3,000 g and 4°C; the serum was stored at −80°C until analysis. The undiluted sample was used to measure the concentration of hsFLT1 (BRAHMS sFlt-1 KRYPTOR assay) with a BRAHMS KRYPTOR compact PLUS analyzer based on time-resolved amplified cryptate emission (TRACE® technology; Thermo Fischer Scientific), according to the manufacturer's protocol (FGR $n = 10$, control $n = 3$, Dox control $n = 6$). The detection limit for hsFLT1 was assessed at 22 pg/ml. The sensitivity of the functional assay, detected by interassay precision of a 20% coefficient of variability (CV), has been assessed to be lower than 29 pg/ml for hsFLT1.

Mouse Angiogenesis Antibody Array

Approximately 20 mg of frozen placenta was homogenized in radioimmunoprecipitation assay (RIPA) protein extraction buffer [50 mM Tris/HCl, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM ethylenediaminetetraacetic acid (EDTA)]. The protein content was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Murine angiogenesis-related proteins were simultaneously detected with a Proteome Profiler Angiogenesis Antibody Array according to the manufacturer's protocol (R&D Systems, Minneapolis, MN USA). In principle, selected capture antibodies for each of 53 different angiogenesis proteins have been spotted in duplicate on nitrocellulose membranes. For protein detection, a total of 50 μg

TABLE 2 | Oligonucleotides used for genotyping, sex determination and gene expression analysis in hsFLT1/rtTA mouse model.

Gene	NCBI number	Primer sequence (5′→3′)	Product length (bp)
GENOTYPING			
hsFLT1	XM_017020485.1	for: AATCATTCCGAAGCAAGGTG	221
		rev: TTTCTTCCCACAGTCCCAAC	
rtTA		for: AAAGTCGCTCTGAGTTGTTAT	
		rev-wt: GGAGCGGGAGAAATGGATATG	650
		rev-mut: GCGAAGAGTTTGTCCTCAACC	340
SEX DETERMINATION			
IL-3	NM_010556.4	for: GGGACTCCAAGCTTCAATCA	544
		rev: TGGAGGAGGAAGAAAAGCAA	
Sry	NM_011564.1	for: TGGGACTGGTGACAATTGTC	402
		rev: GAGTACAGGTGTGCAGCTCT	
REFERENCE/HOUSEKEEPING GENE			
Gapdh	XM_011241214.1	for: ACAACTCACTCAAGATTGTCAGCA	121
		rev: ATGGCATGGACTGTGGTCAT	
AMINO ACID TRANSPORTERS			
Slc38a1	NM_001166458.1	for: AGCACAGGCGACATTCTCATC	133
		rev: ACAGGTGGAACTTTGTCTTCTTG	
Slc38a2	NM_175121.3	for: ACAAATGGGTTGTGGTATCTG	92
		rev: CCTAGATTTCTCAGCAGTGACAATG	
FATTY ACID TRANSPORTERS			
Cd36	XM_006535623.2	for: CAGTGCAGAAACAATGGTTGTCT	137
		rev: TGACATTTGCAGGTCTATCTACG	
Fabp3	NM_010174.1	for: CTGTCACCTCGTCGAACTCT	166
		rev: TTTGTCGGTACCTGGAAGCT	
GLUCOSE TRANSPORTERS			
Glut-1	NM_011400.3	for: GCTGTGCTTATGGGCTTCTC	202
		rev: ACACCTGGGCAATAAGGATG	
Glut-3	NM_011401.4	for: GGAGGAGAACCCTGCATATGATA	96
		rev: TGGCTTCATAGTCATCCTTTAGTAAC	
LABYRINTHINE DIFFERENTIATION MARKERS			
Cx26	NM_008125.3	for: ATGCTACGACCACCACTTCC	194
		rev: TACGGACCTTCTGGGTTTTG	
Gcm1	NM_008103.3	for: TGCTCACCTATGGCTCTCCT	201
		rev: AAAATTCTGCCSAGCCCTTT	
FETAL ENDOTHELIAL CELL MARKER			
Cd31	NM_008816.3	for: ATGACCCAGCAACATTCACA	200
		rev: CACAGAGCACCGAAGTACCA	
TROPHOBLAST GIANT CELL MARKERS			
Ctsq	NM_029636	for: GTGATCTGAGGCAGTAGTGGTC	180
		rev: GTACTTCTTCCTCCGGACTGTATA	
PLAP	XM_006538500.2	for: TGAGGGCAATGAGGTCACAT	161
		rev: CCTCTGGTGGCATCTCCTTA	
Prl3d1	NM_008864	for: TGGAGCCTACATTGTGGTGGA	131
		rev: TGGCAGTTGGTTTGGAGGA	
Prl3b1	NM_008865.3	for: AGCAGCCTTCTGGTGTTGTC	197
		rev: TGTGACACCACAATCACACG	
Prl2c2	NM_011118	for: AGGAGCCATGATTTTGGATG	203
		rev: ACCAGGCAGGGTTCTTCTTT	
SPONGIOTROPHOBLAST AND GLYCOGEN CELL MARKERS			
Cx31	NM_008126	for: GTCTACTAGCGCTGGGATGG	227
		rev: GTGCCAAACCTTCTCATGGT	

(Continued)

TABLE 2 | Continued

Gene	NCBI number	Primer sequence (5′→3′)	Product length (bp)
Cx31.1	NM_008126	for: CCCTCTTTGCTTGTGGTCAT	151
		rev: CCTTGAACGAGAGGCTGAAG	
Igf2	NM_010514	for: CGTTTGGCCTCTCTGAACTC	155
		rev: GACGACTTCCCCAGATACCC	
Pcdh12	NM_017378.2	for: CTTCACCTCATCACGCTCAA	197
		rev: TGCCCTCTGTCCTCTGCTAT	
Tpbpa	NM_009411	for: CCAGCACAGCTTTGGACATCA	116
		rev: AGCATCCAACTGCGCTTCA	
ANGIOGENESIS MARKERS			
Flk-1	NM_001363216.1	for: GGCGGTGGTGACAGTATCTT	162
		rev: GTCACTGACAGAGGCGATGA	
m(s)Flt-1	NM_001363135.1	for: TATAAGGCAGCGGATTGACC	159
		rev: TCATACACATGCACGGAGGT	
Flt-4	NM_008029.3	for: GTGGCTGTGAAGATGCTGAA	199
		rev: TGACACGCAAGAAGTTGGAG	
Plgf	XM_011244016.1	for: CGTCCTGTGTCCTTCTGAGT	200
		rev: CCTCTTCCTCTTCCCCTTGG	
Vegfa	NM_001025257.3	for: CAGGCTGCTGTAACGATGAA	140
		rev: GCATTCACATCTGCTGTGCT	
Vegfb	NM_011697.3	for: AACACAGCCAATGTGAATGC	157
		rev: GGAGTGGGATGGATGATGTC	
Vegfc	NM_009506.2	for: CAAGGCTTTTGAAGGCAAAG	159
		rev: TCCCCTGTCCTGGTATTGAG	
Vegfd	NM_001308489.1	for: CAACAGATCCGAGCAGCTTC	155
		rev: AAAGTTGCCGCAAATCTGGT	
PROAPOPTOTIC MARKERS			
Bad	NM_001285453.1	for: GGAGCTTAGCCCTTTTCGAG	166
		rev: GCTTTGTCGCATCTGTGTTG	
Casp9	NM_001355176.1	for: GATGCTGTCCCCTATCAGGA	151
		rev: CGATGTACCAGGAGCCACTT	
HYPOXIA-INDUCIBLE MARKERS			
Nos3	XM_006535639.3	for: GACCCTCACCGCTACAACAT	209
		rev: CTGGCCTTCTGCTCATTTTC	
Hif1α	NM_001313920.1	for: TCAAGTCAGCAACGTGGAAG	198
		rev: TATCGAGGCTGTGTCGACTG	
Hmox1	NM_010442.2	for: CACGCATATACCCGCTACCT	175
		rev: CCAGAGTGTTCATTCGAGCA	
Cited2	NM_010828	for: CTAGGGCAGCGGAGGAAAAG	176
		rev: TTCTGCTCGGAACACCGAAG	

A, adenine; Bad, Bcl-2–associated death promoter; bp, base pair; Casp9, caspase 9; Cd31, cluster of differentiation 31; Cited2, Cbp/P300 Interacting Transactivator With Glu/Asp Rich Carboxy-Terminal Domain 2; Ctsq, cathepsin Q; C, cytosine; Cx26, connexin 26; Fabp3, fatty acid binding protein 3; Flk-1, fetal liver kinase 1; Flt-4, Fms-like tyrosine kinase 4; for, forward; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Gcm1, glial cell missing 1; Glut-1, glucose transporter 1; G, guanine; Hif1α, hypoxia-inducible factor-1alpha; Hmox1, Heme Oxygenase 1; hsFLT1, human soluble fms-like tyrosine kinase 1; Igf2, insulin-like growth factor 2; IL-3, interleukin-3; mut, mutant; NCBI, National Center for Biotechnology Information; Nos3, Nitric Oxide Synthase 3; Pcdh12, Protocadherin 12; PLAP, Placental Alkaline Phosphatase; Plgf, Placental Growth Factor; Prl3b1, prolactin family 3; subfamily b, member 1; rev, reverse; rtTA, reverse tetracycline-controlled transactivator; Slc38a1, Solute Carrier Family 38 Member 1; Sry, sex determining region Y; Tpbpa, Trophoblast-specific protein alpha; T, thymine; Vegfa, Vascular Endothelial Growth Factor A; wt, wild-type.

protein of a pooled sample of each condition (control $n = 5$, Dox control $n = 5$, FGR het $n = 5$ and FGR hom $n = 5$; each 10 μg) was diluted and mixed with a cocktail of biotinylated detection antibodies. The sample/antibody mixture was then incubated with the array at 4°C over night. Streptavidin-horseradish peroxidase and chemiluminescent detection reagents were added, and chemiluminescence was detected with ChemiDoc™ XRS+ System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Pixel intensity for each spot was measured with ImageJ (National Institutes of Health, Bethesda, MD, USA) and normalized to

negative and reference spots. Normalized intensities of the pair of duplicate spots representing each angiogenesis-related protein were determined and the most relevant proteins were presented.

Serum Metabolome Detection

Maternal serum was obtained as described above. Serum metabolome analysis was performed by Biocrates with the Biocrates AbsoluteIDQ p180 Kit at their facility (Biocrates Life Sciences AG, Innsbruck, Austria), as described previously (26). The following maternal groups were analyzed: FGR group, $n = 6$ [hsFLT1/rtTA, treatment with Dox at 7.5 ($n = 3$) and 10.5 dpc ($n = 3$), respectively]; control group, $n = 6$ [hsFLT1/rtTA, not treated with Dox ($n = 3$) and hsFLT1, treated with Dox at 10.5 dpc ($n = 3$)]. A commercially available direct-flow injection system and a liquid chromatography tandem mass spectrometry (LC-MS/MS) kit was used to analyze 188 available metabolites in plasma samples, including hexose (1), amino acids (21), biogenic amines (21), glycerophospholipids (90), sphingolipids (15), and acylcarnitines (40). Internal standards were pipetted in advance, and a calibration standard mix in seven different concentrations was included in a standardized assay in a 96-well plate, with 10 µl serum in each well. Derivatization was performed with a 5% solution of phenyl isothiocyanate, followed by extraction via the addition of methanol with 5 mM ammonium acetate. The samples were analyzed with an API4000 Qtrap® MS/MS instrument (Applied Biosystems) using a reverse-phase high-performance liquid chromatography (HPLC) column, followed by a direct-flow injection assay.

Histologic and Morphometric Analysis

Formalin-fixed and paraffin-embedded placentas were sectioned at 7 µm and mounted on either standard slides (Engelbrecht Medizin- und Labortechnik GmbH, Edermünde, Germany) or Superfrost Plus Slides (R. Langenbrinck, Emmendingen, Germany). For morphological analysis, sections were stained with hematoxylin and eosin (H&E). Stained slides were scanned with the Aperio CS2 ScanScope slide scanner (Leica, Wetzlar, Germany) at 40×, and images were converted to TIFFs via Image Scope (Version 12.3.2.8013; Leica). Scanned slides were opened by ImageJ with the plugin "bioformats_package.jar."

Morphometric analysis of placental compartments (labyrinth and spongiotrophoblast layer) was performed on three serial sections of three different parts in the proximity of the umbilical cord from each experimental group (FGR wt $n = 8$, control $n = 8$, Dox control $n = 7$). Total placental area was calculated by combining measurements of labyrinth and spongiotrophoblast area; differences in placental compartment composition were measured by the ratio of labyrinth to spongiotrophoblast area as previously described (19).

Immunohistochemical Analysis

Deparaffinized sections were used for immunostaining. Endogenous peroxidase was blocked with H_2O_2 in methanol (1 ml methanol per 25 µl H_2O_2). Antigens were retrieved by boiling sections with citrate buffer for 10 min. After blocking with bovine serum albumin, sections were incubated overnight at 4°C with rat anti-Cd31 (DIA310; 1:20; Dianova, Hamburg, Germany). Bound primary antibody was visualized with goat anti-rat immunoglobulin G horseradish peroxidase (IgG-HRP) secondary antibody (sc-2032; 1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and the Liquid DAB+ Substrate Chromogen System (Dako, Carpinteria, CA, USA).

For placental alkaline phosphatase (PLAP) staining, samples were deparaffinized, rehydrated, and incubated with Nitro Blue Tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega Corporation, Madison, WI, USA) as a substrate for PLAP. Nuclear Fast Red (Sigma-Aldrich) was used for counterstaining. Stained slides were scanned and converted as described above.

Statistical Analysis

Normal distribution was tested with D'Agostino-Pearson omnibus K2 test and Shapiro-Wilk test. Both normality tests could not prove that all of our data were sampled from a Gaussian distribution. Therefore differences between groups were calculated with the Kruskal–Wallis test and Dunn's multiple comparison test. Data are either presented in mean ± standard error of mean or in box and whisker plot. For all statistical tests, a probability value (p-value) of 0.05 or less was indicated with *, $^{**}p < 0.01$ $^{***}p < 0.001$. Outliers were detected performing Grubbs' test (https://www.graphpad.com/quickcalcs/Grubbs1.cfm). Spearman correlation was used to test the association between selected variables. Since correlation is an effect size, the following descriptions of various values of r (Spearman correlation coefficient) were used as a guide to estimate the measured values: $|r| = 0.00$–0.19, no correlation; $|r| = 0.20$–0.39, weak correlation; $|r| = 0.40$–0.59, moderate; $|r| = 0.60$–0.079, strong correlation; $|r| = 0.80$–1.0, very strong correlation. Data were analyzed with GraphPad Prism software version 5.01 (GraphPad, La Jolla, CA, USA).

Statistical analysis of the serum metabolome detection was performed with MetaboAnalyst 4.0 [http://www.metaboanalyst.ca; (27)]. Row-wise normalization was performed with a pooled sample from the control group, and column-wise normalization was performed by log2 transformation of the data. Univariate data analysis was performed with a volcano plot with a fold-change threshold of two and with t-tests at a threshold of 0.05, as well as with the Mann-Whitney U-test. Multivariate data analysis was performed with partial least squares discriminant analysis (PLS-DA) and heat map analysis (Top 25) for visualizing the metabolic differences between FGR and control dams.

RESULTS

The hsFLT1/rtTA Mouse Model Led to Ubiquitous Overexpression of hsFLT1

We used transgenic hsFLT1/rtTA mice, in which hsFLT1 can be ubiquitously induced at several time points during pregnancy and is expressed in dams and fetuses or only in dams (depending on the fetal *rtTA* genotype, as described in the mating scheme) (**Figure 1B**).

At 18.5 dpc, hsFLT1 was detected in the hsFLT1/rtTA dams treated with Dox at early or midgestation (7.5/10.5 dpc; FGR group) but not in the two control groups, with the following

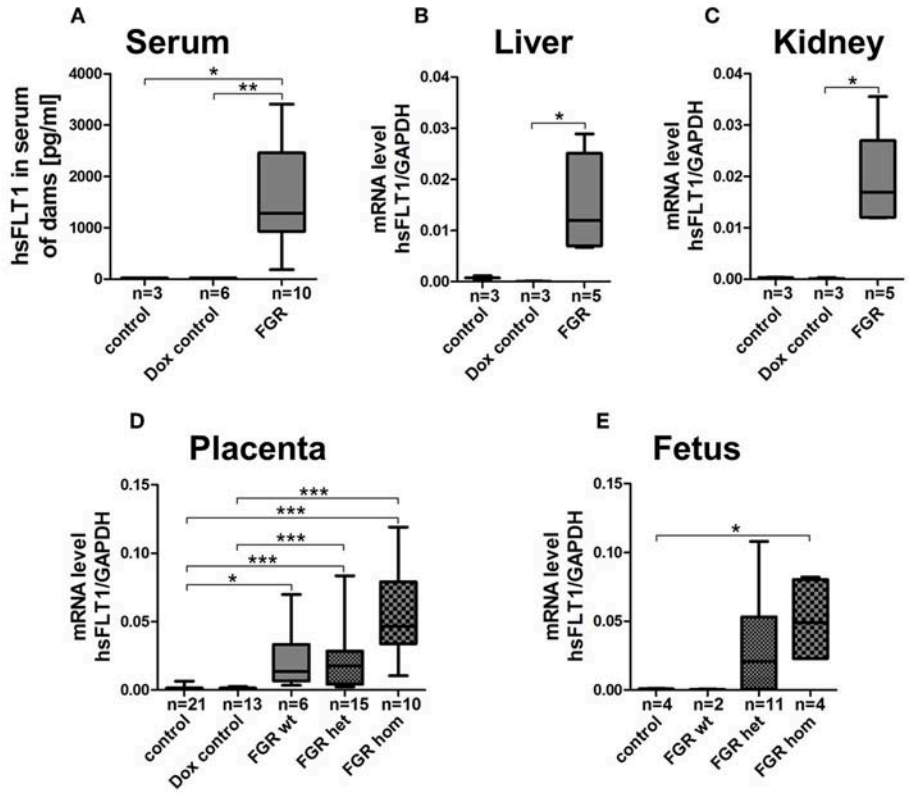

FIGURE 2 | Expression level of hsFLT1 protein in maternal blood serum as measured by ELISA **(A)** and expression of hsFLT1 mRNA in maternal liver **(B)** and kidney **(C)** as well as in placentas **(D)** and fetuses **(E)** as determined by qPCR of the hsFLT1/rtTA mouse model. Expression of human soluble fms-like tyrosine kinase 1 (hsFLT1) occurred only in the mice that exhibited induced hsFLT1/rtTA (reverse tetracycline-controlled transactivator) after treatment with doxycycline (+Dox) (FGR group) and that also exhibited higher placental and fetal *hsFLT1* expression depending on the fetal *rtTA* genotype. Low *hsFLT1* expression was detectable in FGR wild-type (wt) placentas and no *hsFLT1* expression in FGR wt fetuses or in the control and Dox control groups. Samples were obtained from complete placentas and fetuses and from maternal livers and kidneys at day 18.5 post coitum (dpc). Measured mRNA levels were normalized to *Gapdh*. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, as determined by the Kruskal–Wallis test and Dunn's *post hoc* test.

hsFLT1 serum levels: FGR, 1587 ± 294 pg/ml compared to <22 pg/ml for control ($p < 0.05$) and dox control group ($p < 0.01$) (**Figure 2A**). Also, high levels of *hsFLT1* mRNA were found in liver and kidney tissue from the FGR dams, indicating ubiquitous maternal hsFLT1 overexpression (**Figures 2B,C**). In addition, elevated *hsFLT1* transcript levels were detected in FGR placentas (FGR wt, het and hom). The placental *hsFLT1* transcript expression strength was associated with the fetal *rtTA* genotype (**Figure 2D**). The highest level of *hsFLT1* expression was detected in the FGR hom placentas, with lower levels in FGR het and wt placentas and no expression in placentas from either control group or Dox control group. The same held true for FGR hom and FGR het fetuses, because they possess both components of the Tet-On system (the r*tTA* and *hsFLT1* allele) that is necessary for hsFLT1 induction (**Figure 2E**). In this case, hsFLT1 could be induced by Dox transfer via umbilical cord blood. The FGR wt placentas exhibited only a weak hsFLT1 expression due to the maternal part of the placenta (**Figure 2D**) and FGR wt fetuses (**Figure 2E**) exhibited no *hsFLT1* expression because they lack the *rtTA* gene.

Induction of hsFLT1 in hsFLT1/rtTA Pregnant Mice Resulted in FGR

Since we found no obvious differences in inducing hsFLT1 expression between treating with Dox at 7.5 dpc (at ectoplacental cone formation) or at 10.5 dpc (at the beginning of placental differentiation; **Figure S1**), two important reproductive stages in placental development, we present here the combined data collected at both time points, defined as early to midgestation (**Figures 2**, **3**).

Using the hsFLT1/rtTA mouse model, we evaluated fetal and placental weight, litter size per dam, amount of resorption and growth retardation, and placental efficiency (fetal to placental weight) (**Figure 3**). Maternal overexpression of hsFLT1 with or without fetal overexpression produced various effects on fetal size, depending on the fetal *rtTA* genotype and, therefore, on fetal hsFLT1 expression, categorized as strong (FGR hom; **Figure 3E**), medium (FGR het; **Figure 3D**; both maternal and fetal hsFLT1-overexpression), or mild (FGR wt; **Figure 3C**; maternal hsFLT1 overexpression only). In contrast, fetal size was normal in the control groups that did not express hsFLT1 (**Figures 3A,B**). Thus,

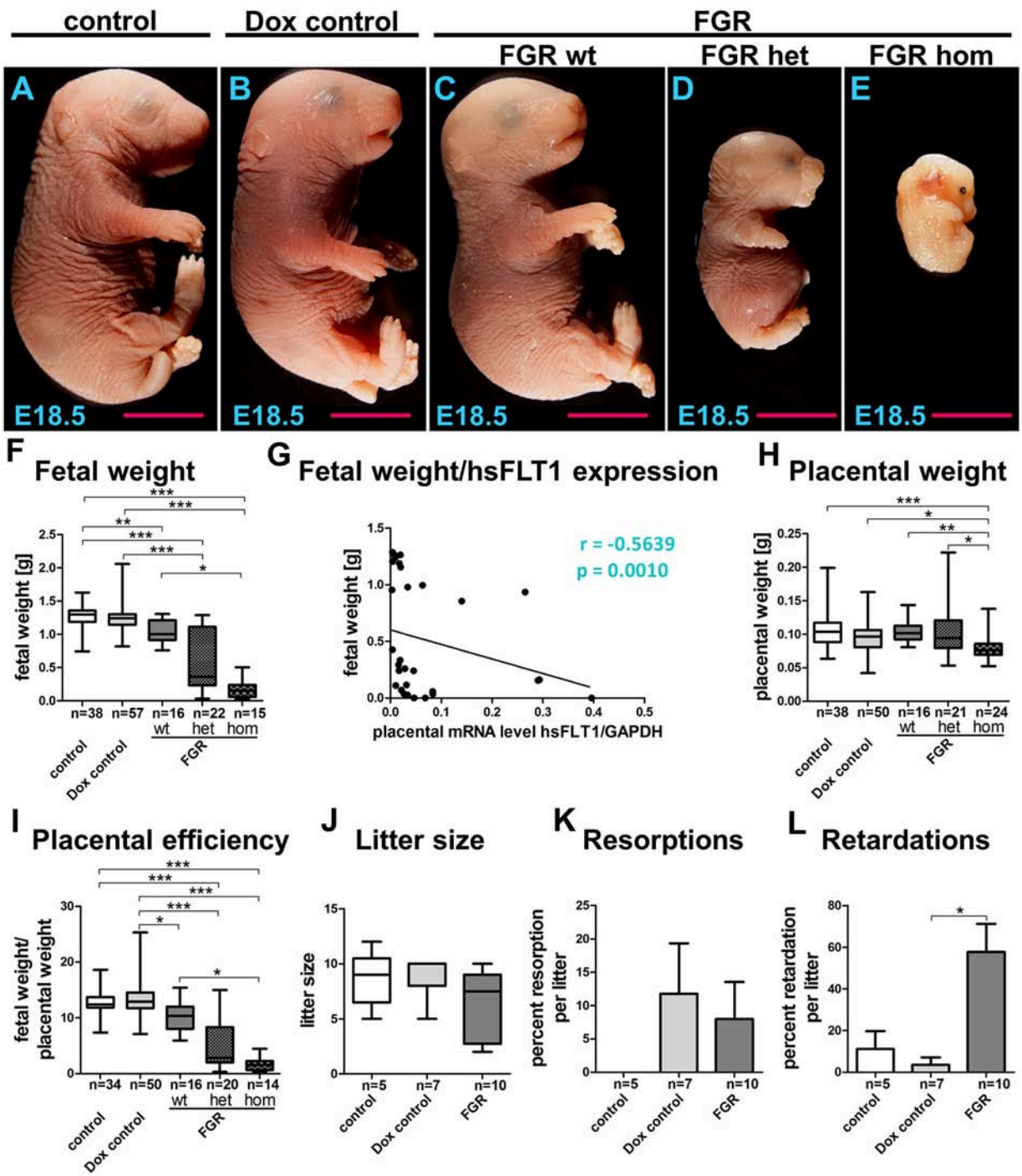

FIGURE 3 | Phenotype of the hsFLT1/rtTA mouse model. Analysis of fetal outcome (fetal weight, litter size, resorptions, and retardations) and the placental phenotype (placental weight, placental efficiency) at day 18.5 post coitum. **(A–E)** Consequences of ubiquitous maternal and/or fetal overexpression of human soluble fms-like tyrosine kinase 1 (hsFLT1) in fetal growth restriction (FGR) homozygous (hom), FGR heterozygous (het), or FGR wild-type (wt) fetuses, showing strong **(A)**, medium **(B)**, or mild **(C)** effects on fetal size in contrast to the control group, which did express hsFLT1 **(D)**. Treating the dam with doxycycline (Dox) (single hsFLT1 mice) produced no negative effects on fetal size **(E)**. These observations were confirmed by analysis of fetal weights **(F)** and by the correlation of fetal weight to placental hsFLT1 expression **(G)**. Placental weight was decreased only in the FGR hom group upon the highest expression of hsFLT1 in fetus and dam **(H)**, whereas placental efficiency (fetal weight/placental weight) is reduced in each FGR group in association with the corresponding hsFLT1 expression levels **(I)**. Litter size **(J)** and number of resorptions **(K)** did not vary between mouse cohorts; however, signs of retardation, such as cyanosis or demise of the fetus, increased in frequency upon hsFLT1 expression **(L)**. Data show hsFLT1/rtTA (FGR) or single hsFLT1 (Dox control) mice treated with Dox at early to midgestation and untreated controls. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by the Kruskal–Wallis test with Dunn's *post hoc* test. Correlation analysis was performed with Spearman's rank correlation coefficient.

treating the dam with Dox (single hsFLT1 mice) exerted no negative effects on fetal size. These observations were confirmed by analysis of fetal weights (**Figure 3F**).

It is shown by us that in a dietary mouse model sex-specific placental differences occurred (28). In the current study, the influence of hsFLT1 on fetal outcome seemed to be sex-specific, with a stronger impact on female fetuses than on male fetuses (**Figure S2A**). However, in the FGR hom and FGR wt groups, fewer male fetuses could be weighed and this difference in numbers may have influenced the ratio between male and female weight outcome (**Figure S2C**). Statistical analysis found a moderate negative correlation between fetal body weight and placental hsFLT1 expression ($r = -0.5639$; $p = 0.0010$) (**Figure 3G**). Also, we found a strong correlation between reduced fetal weights and increased fetal hsFLT1 expression ($r = -0.6691$; $p = 0.0033$) (**Figure S3A**).

Placental weight was decreased in the FGR hom group only at the highest levels of hsFLT1 expression in fetus and dam (**Figure 3H**). In contrast, placental efficiency (fetal weight/placental weight) was reduced in each FGR group in association with the corresponding hsFLT1 expression levels: we found a weak negative correlation between placental efficiency and placental hsFLT1 expression ($r = -0.2439$; $p = 0.24$) and a moderate negative correlation between placental weight and fetal hsFLT1 expression ($r = -0.4059$; $p = 0.1188$) (**Figure 3I**; **Figures S3B,C**). In addition, reduced placental efficiency affects female fetuses more frequently than male ones, as seen before in the differences in fetal weights (**Figure S2B**). Litter size (**Figure 3J**) and resorption (**Figure 3K**) did not vary between the experimental groups; however, Dox treatment seemed to be associated with smaller litter size upon hsFLT1 expression and with a larger number of resorptions, independent of hsFLT1 expression. Signs of retardation, such as cyanosis were frequently observed in the litters of all hsFLT1–expressing dams (FGR group) (**Figure 3L**).

Elevated hsFLT1 Levels Led to Severe Changes in Maternal and Fetal Vascularization

FGR is often associated with placental dysfunction and malnutrition (1). Hence, we asked whether the anti-angiogenic factor hsFLT1 affects placental development, vascularization, and function. Histologic analysis of the hsFLT1–expressing placentas in the FGR hom and het groups showed a severe impairment in placental structure due to extremely enlarged blood-filled spaces, called lacunas (**Figures 4J–O**); this impairment did not appear in control placentas (**Figures 4A–F**). The largest alterations in placental morphology were found in the FGR hom group and were associated with high placental hsFLT1 levels. The large lacunas found in the FGR hom and het placentas appeared to sprout from the chorionic plate into the labyrinth, which is responsible for nutrient exchange (**Figures 4K,L,N,O**). The FGR wt placentas, which are characterized by a weak expression of hsFLT1 due to the maternal part, exhibited a morphological phenotype different from that of FGR hom/het placentas and control placentas, in which the labyrinth compartment was appropriately differentiated, with branched villi containing fetal blood vessels and trophoblast layers lining the longitudinally arranged maternal sinusoids (**Figures 4G–I**). To analyze this more deeply we assessed FGR wt placentas for total placental area as well as labyrinth and spongiotrophoblast layer (**Figure 5**). Total placental area (**Figure 5C**) and labyrinth area (**Figure 5A**) tended to be slightly decreased in the FGR wt placentas in comparison to controls and in addition the labyrinth to spongiotrophoblast ratio (**Figure 5D**) was also reduced in FGR wt placentas compared to controls.

To determine whether the dilated vessels in the FGR het and hom placentas were of maternal or fetal origin, we immunostained paraffin sections for the fetal endothelial cell (EC) marker cluster of differentiation 31 (Cd31; **Figures 6A,C,E,G,I**). Cd31 staining showed that the number of fetal vessels was lower in the hsFLT1–expressing placentas (FGR hom and het) (**Figures 6G,I**) than in control placentas (**Figures 6A,C**), with the strongest reduction in the FGR hom placentas. Moreover, in FGR hom and FGR het placentas, single ECs were detected in the labyrinthine compartment, but they did not form appropriate fetal capillaries (**Figures 6G,I**). The Cd31 signal in the stained FGR wt placentas (**Figure 6E**) was similar to that in the control placentas (**Figures 6A,B**). We also found lower levels of *Cd31* in hsFLT1–expressing FGR hom and FGR het placentas than in control placentas at the transcript level (**Figure 7A**).

Staining for PLAP, which is exclusively present in the sinusoidal trophoblast giant cell (S-TGC) subtype and which lines the maternal sinusoids in the labyrinth (**Figures 6B,D,F,H,J**), demonstrated that the observed large lacunas in the hsFLT1–expressing placentas (FGR hom and FGR het) were maternal sinusoids (**Figures 6H,J**). The FGR wt placentas exhibited maternal sinusoids that were more longitudinally arranged and slightly larger or more swollen (**Figure 6F**) than those exhibited by the other groups. All maternal sinusoids in the control placentas exhibited a normal phenotype (**Figures 6B,D**). *PLAP* mRNA expression was also lower in hsFLT1–expressing placentas (FGR hom and het) than in control placentas (**Figure 7A**), whereas the levels of an additional marker of S-TGCs, cathepsin Q (*Ctsq*), were only moderately reduced in FGR hom placentas and were unchanged in the experimental groups (**Figure 7A**).

Elevated hsFLT1 Levels Led to Severe Changes in Placental Differentiation in the Labyrinthine Compartment, as Characterized by Inhibition of Vegf Signaling

Because we found that hsFLT1 expression exerted a strong effect on changes in the placental labyrinthine compartment, we also quantified syncytiotrophoblast differentiation marker genes of the labyrinth, such as the leading transcription factor glial cell missing 1 (*Gcm1*) and the gap junction protein connexin 26 (*Cx26*), which is located between the two syncytiotrophoblast layers (**Figure 7A**). We found lower transcript levels of both differentiation marker genes (*Gcm1* and *Cx26*) in the FGR hom and het placentas than in control placentas, corresponding to

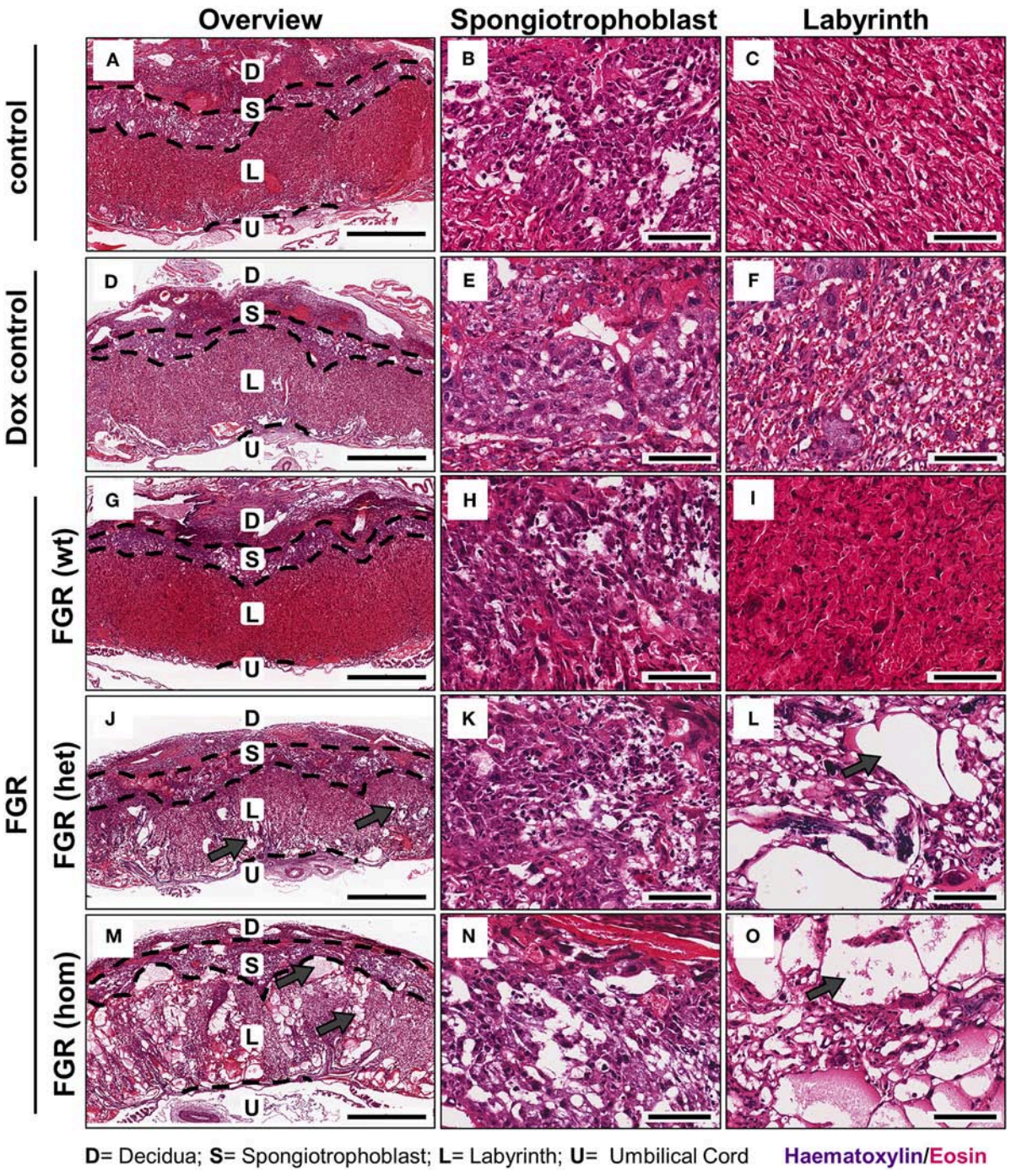

FIGURE 4 | Placental morphology at day 18.5 post coitum in the hsFLT1/rtTA mouse model. Placentas from the various experimental groups were collected and stained with hematoxylin and eosin (H&E). The following groups are shown either in 2× overview (left) or in a 20× detailed structure view of spongiotrophoblast (middle) and labyrinth (right): control [$n = 16$ **(A–C)**] and doxycycline (Dox) control placentas [$n = 12$ **(D–F)**], fetal growth restriction wild-type [FGR wt; $n = 9$ **(G–I)**], FGR heterozygous het; $n = 3$ **(J–L)** and FGR homozygous placentas [hom; $n = 10$ **(M–O)**]. High-expressing human soluble fms-like tyrosine kinase 1 (hsFLT1) FGR hom and FGR het placentas (maternal and placental hsFLT1 expression) exhibited enlarged blood-filled spaces (lacunas, indicated by gray arrows) within the entire placenta, but low-expressing hsFLT1 FGR wt placentas (with maternal hsFLT1 expression only), non-expressing hsFLT1 control placentas, and Dox control placentas did not exhibit such lacunas [**(A,D)** compared to **(G,J,M)**]. The lacunas were located not only in the spongiotrophoblast [**(B,E)** compared to **(H,K,N)**], but also in the labyrinth of hsFLT1–expressing placentas [**(C,F)** compared to **(I,L,O)**]. Interestingly, the FGR wt placentas exhibited a phenotype with a morphology between those of FGR hom/het placentas and control placentas, in which the labyrinth compartment is more densely characterized by an intense staining pattern. Scale bar 2× overview = 1,000 μm; 20× details =100 μm. D, decidua; L, labyrinth; S, spongiotrophoblast; U, umbilical cord.

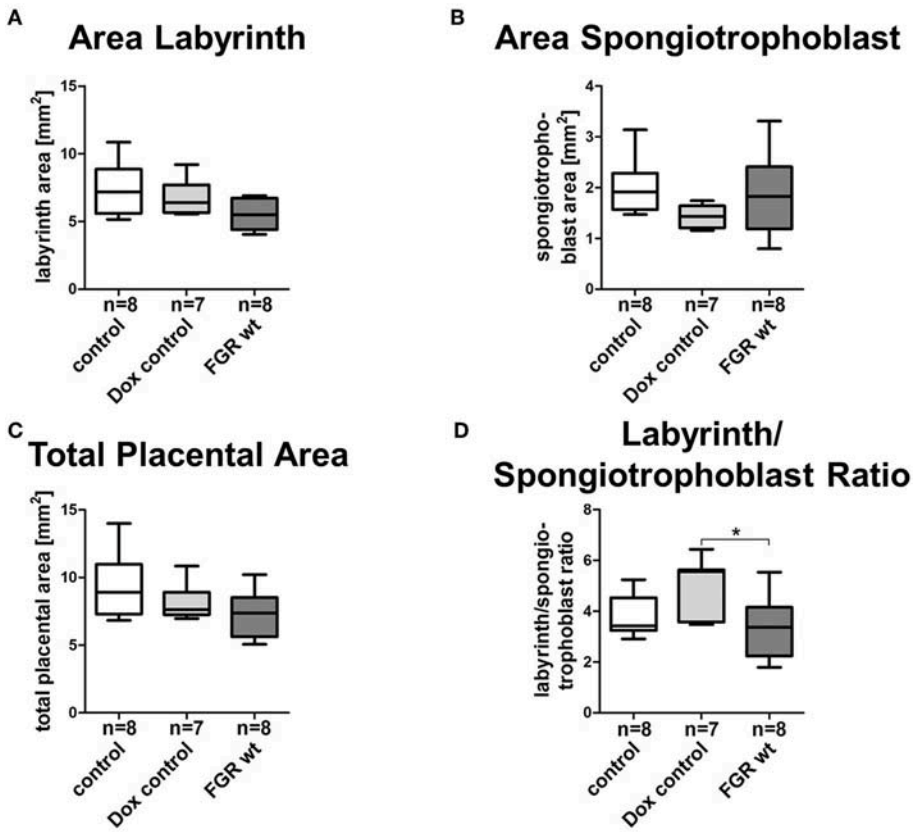

FIGURE 5 | Morphometric analysis of placentas in the hsFLT1/rtTA mouse model. Analysis of the labyrinthine **(A)**, spongiotrophoblast **(B)**, and total placental area **(C)** as well as labyrinth to spongiotrophoblast area ratio **(D)** of human soluble fms-like tyrosine kinase 1 (hsFLT1) expressing fetal growth restriction wild-type placentas (FGR wt; $n = 8$), compared to control ($n = 8$), and doxycycline (Dox) control ($n = 7$) placentas. FGR wt placentas showed slight reduction in labyrinth and total placental area and labyrinth to spongiotrophoblast ratio compared to both control groups, whereas spongiotrophoblast area was slightly increased. Data is presented in box and whisker plot. *$p < 0.05$ as determined by the Kruskal–Wallis test with Dunn's *post hoc* test.

the hsFLT1 levels in each experimental group. We also analyzed various markers on transcript level for spongiotrophoblast and glycogen cells, which are mainly involved in endocrine regulation. These markers include the prolactin *Prl3d1* as a marker for parietal trophoblast giant cells (P-TGCs), *Cx31*, and trophoblast-specific protein alpha (*Tpbpa*), all of which are upregulated upon hsFLT1 expression (refer to **Figure S4A**).

We also investigated transcriptional changes in the endogenous Vegf signaling cascade in the placentas of the various experimental groups. We analyzed most of the relevant genes in this pathway, such as the murine/intrinsic variants of *sFlt-1*/*Flt-1* (*m(s)Flt-1*); fetal liver kinase 1 (*Flk-1*); the ligands *Plgf*, *Vegfa*, and *Vegfb*; and the proapoptotic markers downstream of Flk-1, such as caspase 9 (*Casp9*) and Bcl-2–associated death promoter (*Bad*) [reviewed by Koch and Claesson-Welsh (29)] (**Figure 7B**). The mRNA levels of murine *sFlt-1*/*Flt-1* and its ligands *Plgf*, *Vegfa,* and *Vegfb* were higher in the FGR group, primarily in the FGR hom group, than in the control groups. In contrast, the levels of *Flk-1*, which is located on fetal ECs (30), are lower in hsFLT1–expressing placentas (FGR hom and FGR het) than in control placentas. In contrast, other Vegf receptors and Vegf isoforms, such as Fms-related tyrosine kinase 4 (*Flt-4*), *Vegfc*, and *Vegfd*, were not regulated by hsFLT1 overexpression (**Figure S4A**).

To analyze the growth factors of the Vegf signaling cascade also on protein level we used a Proteome Profiler™ Mouse Angiogenesis Antibody Array to simultaneously detect 53 angiogenesis-related proteins in a single sample. We found a downregulation of total Vegf, Vegfb in particular, and Plgf in the FGR hom and het placentas compared to the controls (**Figure 7C**). In addition, the Angiogenesis Antibody Array exhibits an upregulation of the angiogenesis-related proteins tissue factor, Serpin E1, and Serpin F1 upon hsFLT1 expression in FGR hom and het placentas compared to both control groups (**Figure S4B**).

Furthermore, we found that the proapoptotic molecules *Bad* and *Casp9* are strongly increased upon hsFLT1 upregulation (**Figure 7B**). Both factors signal downstream of Flk-1 and have been shown to be strongly negatively regulated by Vegfa (29). hsFLT1–expressing FGR hom and het placentas exhibited increased transcript levels of both proapoptotic markers.

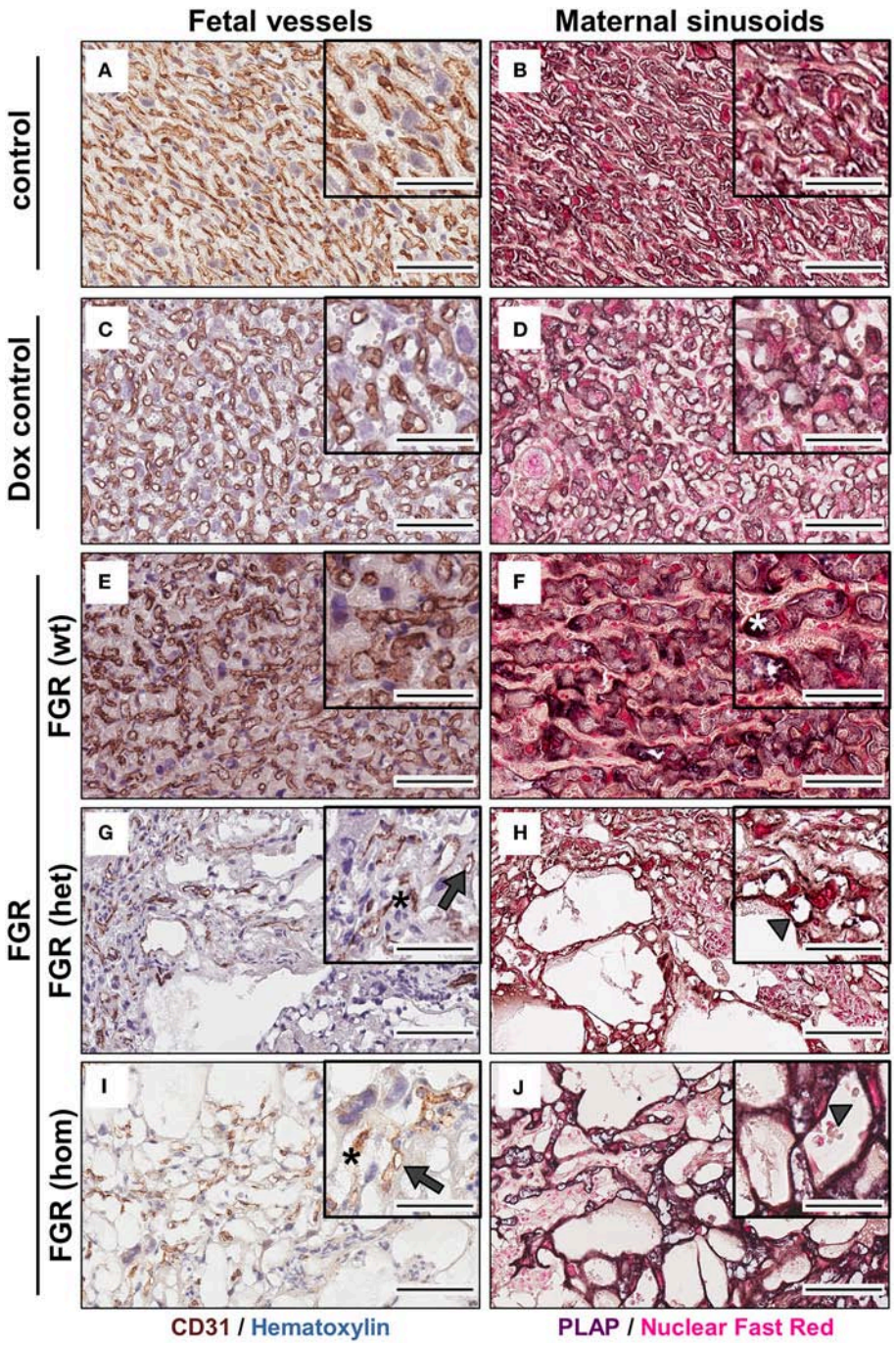

FIGURE 6 | Analysis of fetal (left) and maternal (right) vascularization in the placental labyrinth in the hsFLT1/rtTA mouse model. Immunohistochemical staining of Cd31 (brown staining) in the labyrinth area indicates fewer fetal vessels (indicated by gray arrows) and inadequate formation of blood spaces (indicated by black asterisks) in high-expressing human soluble fms-like tyrosine kinase 1 (hsFLT1) fetal growth restriction homozygous (FGR hom; $n = 10$) **(I)** and FGR heterozygous (het; $n = 3$) **(G)** placentas (maternal and fetal hsFLT1 expression) than in low-expressing hsFLT1 FGR wt [$n = 9$ **(E)**] (maternal expression only) and non-expressing hsFLT1 control ($n = 16$) and doxycycline (Dox) control ($n = 12$) placentas **(A,C)**. For Cd31 staining nuclei are counterstained in blue. Cells lining dilated vessels (lacunas) in high-expressing hsFLT1 FGR hom [$n = 10$ **(J)**] and FGR het [$n = 3$ **(H)**] placentas exhibited placental alkaline phosphatase (PLAP) activity (dark purple staining), a finding indicating the presence of sinusoidal trophoblast giant cells (S-TGCs); therefore, these vessels are characterized as maternal sinusoids (indicated by gray arrowheads). In addition, PLAP-positive vessels in low-expressing hsFLT1 FGR wt placentas [$n = 9$ **(F)**] exhibited a different phenotype, especially for the maternal sinusoids, with more longitudinally arranged and slightly larger sinusoids than in the other groups (indicated by white asterisks). In contrast, the controls [control $n = 16$ **(B)** and Dox control $n = 12$ **(D)**] did not exhibit dilatation of maternal sinusoids. For PLAP staining nuclei are counterstained in light red. Scale bar 20× details = 100 μm and 40× details = 50 μm.

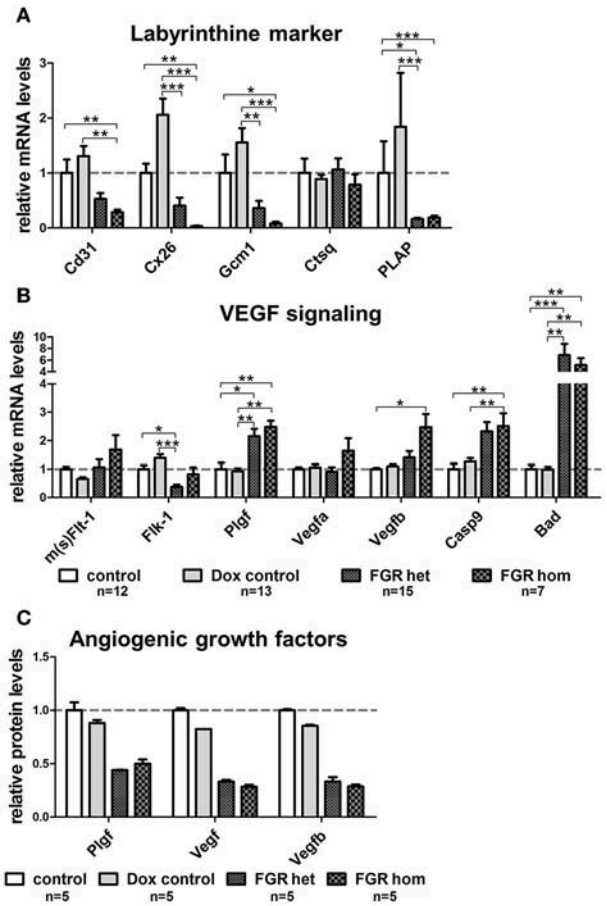

FIGURE 7 | Gene expression analysis of important placental labyrinthine markers and Vegf signaling molecules in the hsFLT1/rtTA mouse model. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) of marker genes in placentas of each group: fetal growth restriction homozygous (FGR hom; $n = 7$), FGR heterozygous (het; $n = 15$), control ($n = 12$), and doxycycline (Dox) control ($n = 13$). **(A)** The mRNA level of fetal endothelial cell marker *Cd31* was lower in placentas expressing human soluble fms-like tyrosine kinase 1 (hsFLT1) (FGR hom; FGR het) than in control placentas (control and Dox control). In addition, the expression of syncytiotrophoblast markers, such as gap junction protein connexin 26 (*Cx26*) and differentiation-promoting transcription factor glial cell missing one (*Gcm1*), is lower in hsFLT1–expressing placentas (FGR hom more pronounced than FGR het) than in control placentas (control and Dox control). The expression of maternal sinusoidal trophoblast giant cell markers, such as placental alkaline phosphatase (*PLAP*), was also lower in hsFLT1–expressing placentas than in control placentas, whereas cathepsin Q (*Ctsq*) expression seemed to be unaffected by hsFLT1 expression in all groups. **(B)** Murine mRNA levels of *sFlt-1* and *Flt-1* (*m(s)Flt-1*) exhibited no clear up- or downregulation between groups (no discrimination between murine *sFlt-1* and murine *Flt-1* possible at the mRNA level), whereas the expression of murine fetal liver kinase 1 (*Flk-1*) was lower in FGR hom and FGR het placentas than in control placentas (control and Dox control). Growth factors, such as placental growth factor (*Plgf*; mainly binding to sFlt-1 and Flt-1), vascular endothelial growth factor A (*Vefga*; mainly binding to sFlt-1, Flt-1, and Flk-1), and *Vegfb* (mainly binding to sFlt-1 and Flt-1), are upregulated at the time of hsFLT1 expression (FGR hom and FGR het), a finding correlating with increasing levels of hsFLT1. Nevertheless, the proapoptotic markers caspase9 (*Casp9*) and Bcl-2–associated death promoter (*Bad*) downstream of Flk-1 are more

(Continued)

FIGURE 7 | highly upregulated upon hsFLT1 expression in FGR hom and FGR het placentas than in either control group (control and Dox control). Samples were obtained from complete placentas at day 18.5 post coitum (dpc). Measured mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh;* except for *Casp9* and *Bad*, which were normalized to *Flk-1*), and control group levels were set at 100% (dotted line). Data is presented as mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ determined by the Kruskal–Wallis test with Dunn's *post hoc* test. **(C)** Protein levels of Plgf, total Vegf, and Vegfb were reduced upon hsFLT1 expression in FGR hom ($n = 5$) and het ($n = 5$) placentas compared to control ($n = 5$) and Dox control group ($n = 5$). Data is presented as mean ± standard deviation.

Overall, we found that, upon hsFLT1 expression, 22 of 34 genes were differently regulated in FGR groups than in control groups, and these differences led to distinct transcriptomic profiles (**Figure S4A**).

Elevated hsFLT1 Levels Altered the Placental Transporter System and Increased the Total Levels of Phosphatidylcholine in Maternal Serum From hsFLT1/rtTA Mice

The expression level of important nutrient transporter genes for the transport of glucose, amino acid, and fatty acid across the placental barrier was screened. For each transport pathway, we analyzed two transporters: for glucose transport, glucose transporter 1 (*Glut-1*; localized in syncytiotrophoblast (ST) layer I and II [reviewed by Winterhager and Gellhaus (20)] and 3 (*Glut-3*; localized in ST layer I); for fatty acid transport, the fatty acid translocase *Cd36* and fatty acid binding protein 3 (*Fabp3*) (both ST layer I and II); and for amino acid transport, solute carrier family 38, members one and two (*Slc38a1* in ST layer I and II); *Slc38a2* only in ST layer II). The expression of all examined transporters was lower in mice expressing induced hsFLT1 (FGR hom and FGR het) than in uninduced controls, with the strongest decrease in the expression of fatty acid transporters (**Figure 8C**).

Since we observed a strongly impaired placental morphology and changes in the transport system, we expected that the induced hsFLT1/rtTA dams (FGR group) and uninduced dams (control group) would exhibit unique metabolomic profiles. Using the Biocrates AbsoluteIDQ® p180 Kit, we investigated 188 endogenous metabolites from five compound classes by tandem mass spectrometry (MS/MS). Metabolites whose levels were below the lower limit of quantification (<LLOQ) were excluded; the remaining 152 metabolites were included in the analysis. To visualize the main metabolic differences between experimental groups, we performed multivariate data analysis with partial least squares discriminant analysis (PLS-DA) and heat map analysis (the 25 top changed metabolites are shown in **Figure 8B**). PLS-DA showed a clear distinction in the variable importance of projection (VIP) scores between the two experimental groups (FGR vs. controls) (**Figure 8A**). A heat map representation of the top 25 modified metabolites showed

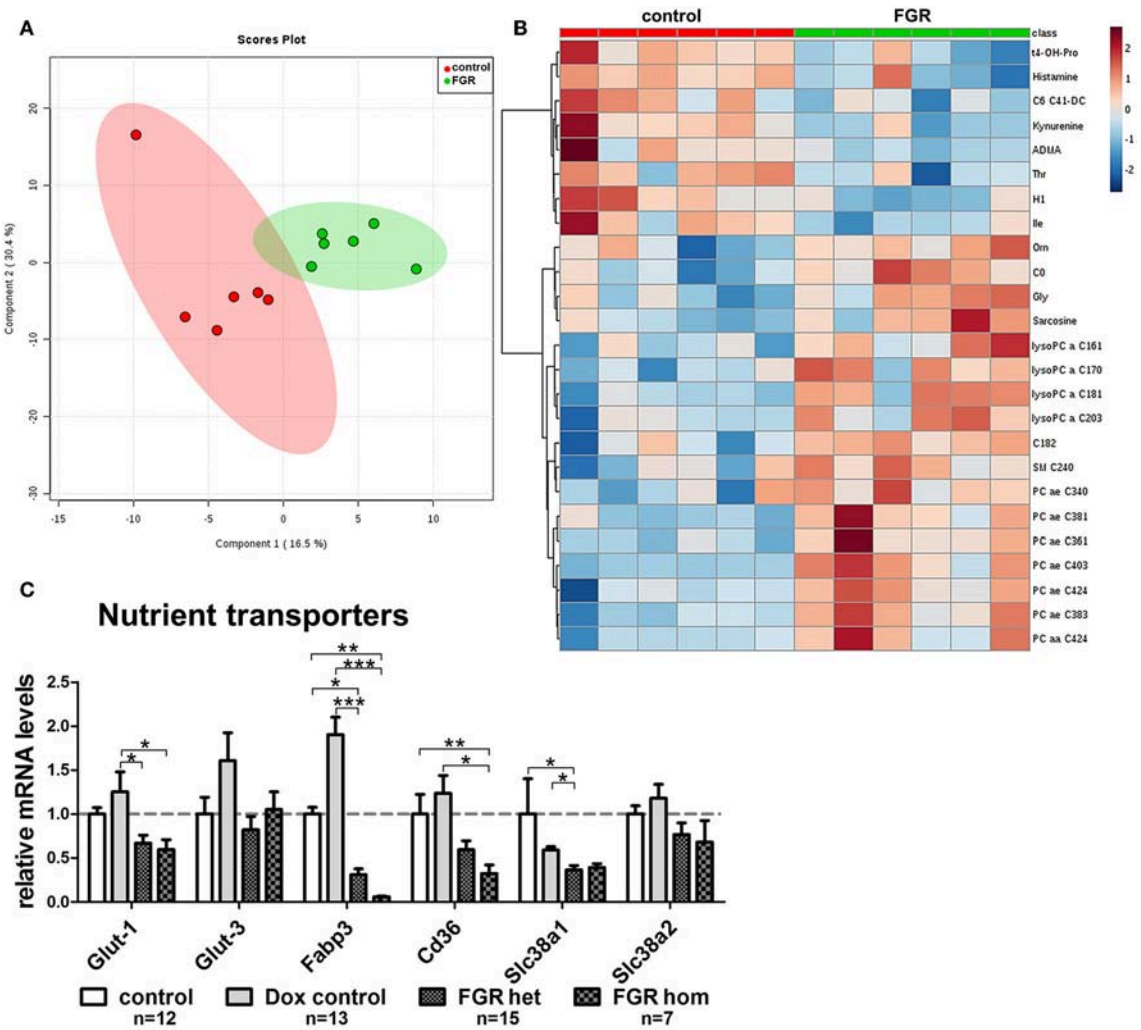

FIGURE 8 | Maternal metabolome analysis and placental expression of nutrient transporter genes in the hsFLT1/rtTA mouse model. **(A)** Supervised partial least squares discriminant analysis (PLS-DA) of 152 metabolites detected the main metabolomic differences in serum from fetal growth restriction dams (FGR; $n = 6$; green dots) and control dams ($n = 6$; red dots). **(B)** Heat map representation of the top 25 modified metabolites in each group ($n = 6$ each) mainly indicated accumulation of lysophosphatidylcholines and phosphatidylcholines in serum from hsFLT1–expressing dams (color-coding intensity in the red spectrum shows an increase in the number of given metabolites, and color intensity in the blue spectrum shows a decrease in the number of the given metabolites). **(C)** Results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of nutrient transporters in placentas from each group: FGR homozygous (hom; $n = 7$), FGR heterozygous (het; $n = 15$), control ($n = 12$), and doxycycline (Dox) control ($n = 13$). The mRNA expression levels of glucose transporters *Glut-1* and *Glut-3*, fatty acid transporters fatty acid binding protein 3 (*Fabp3*) and *Cd36*, and amino acid transporters solute carrier family members one and two (*Slc38a1* and *Slc38a2*) are lower in hsFLT1–expressing placentas (FGR hom and FGR het) than in control placentas (control and Dox control); the strongest decrease was observed in the fatty acid transporters. Samples were obtained from complete placentas at day 18.5 post coitum (dpc). Measured mRNA levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), and the control group was set at 100% (dotted line). Data is presented as mean ± standard error of the mean. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by the Kruskal–Wallis test with Dunn's *post-hoc* test.

distinct metabolic footprints between the FGR group and the control group, with 17 upregulated and eight downregulated metabolites (**Figure 8B**). Most of the upregulated metabolites were lipids, whereas most of the downregulated metabolites were amino acids or biogenic amines. Furthermore, volcano plot analysis, a combination of fold change (FC = 2) and *t*-test results ($p < 0.05$), showed that 13 changed metabolite levels were found in serum from dams (FGR and controls). Ten of these were upregulated, and most were long chain fatty acid phosphatidylcholine (PC)/lysophosphatidylcholines or the amino acid glycine (Gly) and its byproduct/precursor sarcosine (**Figure 8B**; **Table 3**). All three downregulated metabolites were biogenic amines.

Overexpression of hsFLT1 Resulted in Only Small Epigenetic Changes in the Placentas

We have previously shown that overexpression of placenta-specific sFLT1 by lentiviral gene delivery results in small changes in DNA methylation (19). Using the previous lentiviral model, we found DNA methylation of *Igf2-DMR2*, *H19-ICR*,

TABLE 3 | Top 13 regulated metabolites in serum among dams in the hsFLT1/rtTA mouse model.

	Fold change	log2(FC)	*p*–value	-log10(p)
PC ae C40:3	0.27987	−1.8372	0.001	3.1843
PC ae C38:3	0.45671	−1.1306	0.002	2.7442
lysoPC a C18:1	0.449	−1.1552	0.004	2.4119
PC ae C38:1	0.36779	−1.443	0.005	2.2804
Gly	0.35951	−1.4759	0.009	2.0239
PC ae C42:4	0.32027	−1.6427	0.010	2.0139
PC aa C42:4	0.3671	−1.4458	0.012	1.909
Sarcosine	0.44167	−1.1789	0.019	1.7266
lysoPC a C20:3	0.31089	−1.6855	0.024	1.6226
PC aa C40:3	0.40968	−1.2874	0.041	1.3855
Kynurenine	2.1765	1.122	0.010	2.0002
t4-OH-Pro	2.0757	1.0536	0.013	1.9025
Histamine	2.0072	1.0052	0.021	1.6826

aa, diacyl; ae, acyl-alkyl; FC, fold change; Gly, glycine; lysoPC, lyso phosphatidylcholine; p, probability; PC, phosphatidylcholine; t4-OH-Pro; trans-4-Hydroxyproline.

and *LINE1*. Two of the five CpG positions in the *Igf2* gene were hypomethylated, but there was no change in general DNA methylation.

Consequently, in the current study we measured DNA methylation at the same loci: *Igf2-DMR2* (five positions), *H19-ICR* (three positions), and *LINE1* (five positions) (**Figure S5**). However, in the FGR wt placentas we found changes in DNA methylation of *LINE1* at CpG position three, but no further changes in DNA methylation at the other four CpG positions. In addition, no changes in DNA methylation were found for *H19-ICR* and *Igf2-DMR2*.

DISCUSSION

Various sFLT1 mouse models based on adenoviral transduction (31, 32) have been used in FGR/PE research; most of these models exhibited an accumulation of sFLT1 in the liver. The effects of sFLT1 on the maternal endothelium and the maternal organs have been described [reviewed by Lecarpentier and Tsatsaris (17)]. However, less is known about the direct and indirect influence of sFLT1 on placental development and function. Therefore, we generated transgenic inducible hsFLT1/rtTA mice, in which hsFLT1 expression can be ubiquitously induced in dams and fetuses by Dox administration at chosen time points between early gestation and midgestation during pregnancy, as in humans.

Since Dox crosses the placental barrier, hsFLT1/rtTA fetuses can express hsFLT1 just as their dams do. Because of the limitations of inheritance rules, we could not generate pregnancies in these transgenic hsFLT1/rtTA mice during which the dams overexpressed hsFLT1 and the fetuses did not. Therefore, we used hsFLT1/rtTA heterozygous mating to create various levels of hsFLT1 expression in fetuses within a single dam, depending on the fetal *rtTA* genotype (hom, het, or wt). With these three possibilities of hsFLT1 expression, we could discriminate between overexpression of hsFLT1 by dams and by fetuses or placentas and, thus, between the effects of maternal hsFLT1 overexpression and those of fetal/placental hsFLT1 overexpression on both fetus/placenta and mother during pregnancy. The rationale and premise for this hsFLT1 mouse model is that we are able to analyze if hsFLT1—only maternally expressed- also affects the fetus indirectly (by altering mediators) or directly via a transplacental transport of hsFLT1 from the mother to fetal circulation. It would require an active transport mechanism, of hsFLT1 with 120 kDa to cross the placental barrier, because the limit of membrane transfer for proteins via the placenta is believed to be ∼500 Da (33). Kumasawa et al. (18) suggested that hsFLT1 in the mother's circulation can pass through the placenta into the fetus and thus contribute to FGR, but evidence was missing. Anyway, we observed only a very small effect in the wt fetuses on fetal growth. Thus, the amount of hsFLT1 levels not only in the maternal but predominantly in the fetal circulation seems to be important. A placental transfer of hs to the fetus is not yet proven by us or others and would need further investigations in future studies. Furthermore, inducing the hsFLT1 expression directly in the fetus itself allows analyzing direct effects of hsFLT1 on placental development and the possible consequences for the dam to develop heart diseases in later life upon fetal expression of hsFLT1.

In addition to the theory that hsFLT1 is transported across the placenta to the fetus it cannot be excluded that the slight growth restriction of FGR wt fetuses is due to the presence of hsFLT1 in the maternal decidua. The maternal sinusoids which exhibit high levels of hsFLT1 bathe the placenta and could influence changes in placental function, as indicated by reduced placental efficiency and FGR. The association between increased sFLT1 levels in maternal circulation and FGR of the fetuses is already shown in humans (34). The results of hsFLT1/rtTA homozygous mating showed that hsFLT1/rtTA homozygous fetuses did not survive after birth, probably because of strong growth retardation or associated other not known malformations. Thus, the survival rate and the reasons for stillbirth in the FGR groups must be confirmed in experiments focused on fetal outcome.

Focusing on the effect of hsFLT1 on placental development, we found that placental weights were reduced in FGR hom placentas but not in FGR het and wt placentas but a decrease in placental efficiency was found in all FGR groups. In the sFLT1 lentiviral mice (19) both, the fetal weight and the placental weight of sFLT1–transduced mice were reduced. These differences in placental weights could be due to the different model systems because in the lentiviral mouse model hsFLT1 is permanently expressed in the trophoblast cells already from the blastocyst stage onwards.

The placental phenotype described here, with enlarged maternal blood sinusoids and reduced numbers of fetal blood vessels, has not been observed in other sFLT1 mouse models (31, 32, 35). The exclusively maternal overexpression of hsFLT1 in the FGR wt group produced results very similar to those found in the placenta-specific lentiviral mice published by Kumasawa et al. (18) and our group (19). Both groups evidenced a reduced labyrinthine layer and in the study of Kumasawa et al. (18) also

a reduction in Cd31 expression. The observed alterations in the maternal blood spaces by Kumasawa et al. (18) could not be seen in our lentiviral mouse model (19) but in the present study using the hsFLT1 maternally overexpressing mice.

We induced hsFLT1 expression in early to midgestation, at the leading time point of chorioallantoic attachment followed by fetal vasculature branching to form dense villi within the murine labyrinth (8.5–10.5 dpc), processes that are strictly regulated by members of the Vegf, Plgf, fibroblast growth factor (Fgf), and transforming growth factor beta (Tgf-β) families (36, 37). At this time point of gestation, the inhibition of angiogenesis by overexpression of hsFLT1 could have the strongest effect and strengthens the finding that Vegf is required for placental development following 7.5 dpc. This finding was closely associated with the change in expression patterns of Vegf signaling molecules and apoptotic markers in the FGR hom and het placentas. Since we induced hsFLT1 expression upon 7.5 dpc, hsFLT1 could also have influenced the yolk sac function, which is the main source of nutrition to the fetus before 10.5 dpc of pregnancy (38). Thus, we cannot exclude that impaired development of the yolk sac function also contributes to FGR. However, taken into account that the later induction of hsFLT1 on 10.5 dpc where the yolk sac is already differentiated lead to the same effect on fetal and placental growth, we suggest a minor role of yolk sac dysfunction in our model.

The unraveling of hsFLT1–associated impairment of the Vegf signaling cascade showed a strong upregulation of the endogenous binding partners of murine sFlt-1 on transcript level in the placentas: the murine variants of *Plgf*, *Vegfa*, and *Vegfb* exhibited a kind of counterregulation to the increase in hsFLT1, indicating the binding of hsFLT1 to the murine ligands. This could be strengthen by a reduction of Vegf and Plgf protein levels in the FGR hom and het groups maybe causing an impaired Vegf signaling, indicated by mRNA upregulation of Vefg-related apoptosis markers. Indeed, Szalai et al. (32) found that hsFLT1 can bind and sequester murine Plgf *in vivo*. The mRNA expression of membrane-bound Vegf receptor 2 (*Flk-1*), which is the leading receptor for angiogenesis and is expressed by placental/fetal ECs (30, 39), was lower in FGR hom and het placentas than in control placentas. The reduction of *Flk-1* transcript levels was combined with a reduction in *Cd31* transcript levels and in the number of fetal ECs.

Moreover, the mRNA expression of proapoptotic markers such as *Casp9* and *Bad* downstream of Flk-1 was highly upregulated upon hsFLT1 expression in FGR hom and het placentas, a finding that argues for apoptosis of fetal ECs and the consequent reduction in *Cd31* signals and in the number of fetal ECs. Jiang et al. (40) found that sFLT1 mediates oxidative stress on trophoblast cells during PE and thereby increases apoptosis. We hypothesize that the same holds true for fetal ECs. These findings indicate that placental Vegf signaling is impaired, and this impairment probably inhibits placental vessel development. Therefore, we hypothesize that the observed FGR phenotype in the hsFLT1/rtTA fetuses results mainly from impaired Vegf signaling via Flk-1 in the placenta, which is triggered by excessive signaling of the anti-angiogenic molecule hsFLT1 and, as a consequence, by reduced binding of Vegfa and Vegfb to Flk-1. In contrast, other Vegf receptors and Vegf isoforms, such as *Flt-4*, *Vegfc*, and *Vegfd*, seem not to be differentially regulated on mRNA level upon hsFLT1 overexpression.

The enlargement of maternal sinusoids in the labyrinthine compartment in the FGR hom and het placentas could be due to a stasis of maternal blood conditioned by a reduced fetal vascular system that increases maternal blood pressure as a possible reactive response to the necessity to fulfill the nutrient requirements of the fetus. Although maternal hypertension and pathological uteroplacental blood flow have not yet been confirmed in our model, this mechanism resembles PE symptoms in humans.

hsFLT1 exerts a strong influence on labyrinth differentiation combined with a decrease of syncytiotrophoblast markers in relation to placental hsFLT1 levels, such as the glucose-diffusion channel *Cx26* and the differentiation-promoting transcription factor *Gcm1*. Gcm1 is one of the leading transcriptional factors during labyrinthine differentiation; it is expressed by a subset of chorionic trophoblast cells and defines the places at which branch points of fetal vessels in the labyrinth will form (41). The reduced transcript expression of markers for the transporting trophoblast fit very well with the FGR phenotype of the fetuses with the various hsFLT1 levels. In addition, the markers for the spongiotrophoblast and glycogen cells, which are mainly responsible for the production of endocrine factors and regulation, have been shown to be upregulated upon hsFLT1 expression; these markers include prolactin *Prl3d1*, as a marker for P-TGCs, and *Cx31,* and *Tpbpa.* This indicates in addition an altered differentiation of the spongiotrophoblast upon hsFLT1 overexpression. The dysregulation of labyrinthine markers in the hsFLT1/rtTA placentas upon hsFLT1 induction in pregnant mice was in accordance with the expression levels of placental nutrient transporters. Transcript levels of glucose transporters *Glut-1* and *Glut-3*, fatty acid transporters *Fabp3* and *Cd36*, and amino acid transporters *Slc38a1* and *Slc38a2* were reduced in hsFLT1–expressing placentas, with the strongest decrease in fatty acid transporters. These alterations strengthened the hypothesis of a negative effect of hsFLT1-expression on placental nutrient transport, leading to a reduction in the transport of nutrients to the fetus. The reduction in the expression of all types of nutrient transporters and of syncytiotrophoblast markers, as well as the reduction in labyrinthine size indicated a reduction in the number of trophoblast cells in the labyrinth but not a downregulation in the expression of these markers per cell. These observations agree with the findings of previous studies using sFLT1 overexpressing mice, which showed a reduction in the size of the labyrinthine compartment (18, 19), and with those of studies using a Plgf knockout mouse model (42) or a Gcm1-deficient mouse model (41, 43).

Thus, we suggest that, in transgenic hsFLT1/rtTA mice, placental function is seriously impaired by hsFLT1 because of a possible deficiency in the placental exchange of nutrients, in particular fatty acid transport. Lipids as central precursors of bioactive molecules are essential for fetal brain development and fetal weight gain (44–46).

Indeed, the metabolic profile of the serum of hsFLT1/rtTA dams showed a change in the metabolome upon hsFLT1

expression, a finding that indicates an accumulation of lysophosphatidylcholines and phosphatidylcholines in the serum of these dams. These findings fitted to the observed reduced level of expression of fatty acid transporters and placental alkaline phosphatases, an enzyme which dephosphorylates phospholipids, which is necessary before transport of the lipids via the placenta (20). Accumulation of phosphatidylcholines in maternal serum and reduction of placental Fabp3 transcript level was also shown in a sFlt-1 overexpressing adenovirus mouse model by Stojanovska et al. (47). Thus, there is an association between reduced placental nutrient transporter expressions with identified protein classes as a result of a dysfunctional placenta. However, we cannot exclude that other altered signaling pathways not involved in placental transport could contribute in elevation of circulating lipids. That FGR is associated with changes in the plasma metabolome, especially amino acid and fatty acid metabolism, has also been shown by other studies, including studies using a maternal diet mouse model (26, 48).

PE with or without FGR is well-known for its long-term consequences for the fetuses (49). Changes in DNA methylation have been proposed to mediate these long-term effects on the fetus (50). Our model of lentiviral sFLT1 overexpression showed methylation of *Igf2*, an imprinted epigenetically regulated gene, and of *H19*, which is co-localized with *Igf2*. Igf2 transcription is dependent on the methylation status of *H19-ICR*, which is located upstream of the H19 promoter (51). In addition, the average methylation of *LINE1* elements was used as a proxy for DNA methylation in general and can therefore serve as an indicator of total DNA methylation levels (52). The use of the transgenic mouse model overexpressing only hsFLT1 did not lead to further changes in the methylation pattern. The current study found only minor changes in DNA methylation, with only one change in *LINE1* and no changes in *H19* or *Igf2*. This finding may be related to the fact that we induced ubiquitous hsFLT1 expression in both the dam and the fetus, and this ubiquitous expression resulted in much higher levels of hsFLT1 at midgestation, unlike the more pathophysiological levels observed in human PE, in which only the placenta overexpresses sFLT1, as in the lentiviral model (19). Moreover, it is tempting to speculate that under the severe conditions observed here we measured methylation in the surviving cell population; thus, there may be an experimental bias by selective survival.

Taken together, the impaired placental development shown in these hsFLT1/rtTA mice ultimately leads to placental insufficiency and FGR. Changes in the transport mechanism of lipids and other nutrients, in combination with a reduction in the placental vascular system, could have caused the strong decrease in fetal weight during pregnancy found in our study. Thus, we speculate that the alterations triggered by the increase in anti-angiogenesis brought about by hsFLT1 expression not only may strongly affect the maternal cardiovascular system, as shown by Mosca et al. (53, 54), but also may adversely affect the fetus by altering development and function of the placenta as the first fetal organ, increasing the risk of cardiovascular and neurological diseases later in life (55).

CONCLUSION

We introduced a novel stable and reproducible transgenic hsFLT1/rtTA FGR mouse model, in which hsFLT1 overexpression can be ubiquitously induced at several time points during murine pregnancy. Using this model, we were able to discriminate between the effects of hsFLT1 overexpression by the dam and the placenta/fetus and those of overexpression by the dam alone. FGR has developed in all hsFLT1 expressing groups at term, and its severity depended on the hsFLT1 expression strength. In the present study we focused primarily on the consequences of hsFLT1 overexpression on placental development, with a special focus on placental vascularization and nutrient transport. The results indicate the importance of the Vegf/sFlt-1 system in placental development in stages following 7.5 dpc in mice. hsFLT1 inhibits placental differentiation, especially inhibiting fetal capillary branching by reducing Vegf signaling and inducing apoptosis in fetal ECs. Over time, this inhibition could lead to a stasis of maternal blood, promoting dilatation of the maternal sinusoids because of impairment of the fetal vessel system. The altered placental morphology ultimately results in uteroplacental insufficiency, including reduced nutrient transport (predominantly affecting the fatty acid supply), which leads to FGR.

Currently we are determining whether these hsFLT1/rtTA mice also exhibit typical symptoms of PE. This improved model can serve as a tool for further molecular biological investigations of sFLT1–mediated pathophysiology in PE with FGR as well as for the development of maternal diseases. Furthermore, it could yield new treatment options for PE and could be used in follow-up studies of fetal and maternal outcome.

ETHICS STATEMENT

All animal experiments were approved and performed in accordance with the guidelines of the University Hospital Essen, Germany, and with local government approval by the State Agency for Nature, Environment and Consumer Protection, North Rhine-Westphalia (LANUV).

AUTHOR CONTRIBUTIONS

AG, EK, and EW initiated and organized the study. ND and HS designed the hsFLT1 mouse strain. AG, EK, RV, and EW designed the experiments. RV performed the experiments, analyzed the data, and prepared the figures and tables. TP and RV-S performed epigenetic analysis, including discussion of the epigenetic data. AG, FH, AK, RK, TP, VS, RV, and EW discussed the data. AG, RV, and EW wrote the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

FUNDING

Financial support was provided by Mercator Research Center Ruhr (MERCUR) An-2015-0009 to AG; Programm zur internen

Forschungsförderung Essen (IFORES) project grant D/107-81240 to AG (Medical Faculty, University of Duisburg-Essen); German Research Foundation (DFG) GE2223/2-1 to AG and HE 6249/1-2, HE 6249/4-1, and HE 6249/5-1 to FH; and by Netherlands Organization for Health Research and Development (ZonMW) 91211053 to TP. We acknowledge support by the Open Access Publication Fund of the University of Duisburg-Essen.

ACKNOWLEDGMENTS

The authors thank Gabriele Sehn for excellent technical assistance. Moreover, we thank the team of the Central Animal Facility, especially Esra Köser, for their assistance with the mice. We are grateful to Dr. Florence M. Witte for English editing and critical reading of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00165/full#supplementary-material

Figure S1 | Comparison of fetal **(A)** and placental weight **(B)** as well as placental efficiency **(C)** of hsFLT1 expression starting at gestational day (dpc) 7.5 or 10.5 until 18.5 dpc in the hsFLT1/rtTA mouse-model. **(A)** fetal weights: Controls 7.5, $n = 13$, 10.5, $n = 25$; Dox controls 7.5, $n = 32$, 10.5, $n = 25$; FGR wt 7.5, $n = 12$, 10.5, $n = 4$; FGR het 7.5, $n = 3$, 10.5, $n = 19$; FGR hom 7.5, $n = 3$, 10.5, $n = 12$. **(B)** Placental weights: Controls 7.5, $n = 11$, 10.5, $n = 25$; Dox controls 7.5, $n = 30$, 10.5, $n = 20$; FGR wt 7.5, $n = 12$, 10.5, $n = 3$; FGR het 7.5, $n = 3$, 10.5, $n = 18$; FGR hom 7.5, $n = 4$, 10.5, $n = 20$. **(C)** Placental efficiency: Controls 7.5, $n = 11$, 10.5, $n = 23$; Dox controls 7.5, $n = 30$, 10.5, $n = 20$; FGR wt 7.5, $n = 12$, 10.5, $n = 3$; FGR het 7.5, $n = 3$, 10.5, $n = 17$; FGR hom 7.5, $n = 3$, 10.5, $n = 17$. Data is presented as median and interquartile range. Two-Way ANOVA with Bonferroni post tests revealed no changes between the starting time points 7.5 and 10.5 dpc in each experimental condition ($p > 0.05$).

Figure S2 | Sex-specific differences in fetal weight and placental efficiency in the hsFLT1/rtTA mouse model. **(A)** Body weight of mouse fetuses at day 18.5 post coitum (dpc) was lower in females expressing human soluble fms-like tyrosine kinase-1 (hsFLT1) than in males expressing hsFLT1. **(B)** In addition, reduced placental efficiency affected female fetuses more frequently than male fetuses. $^*p < 0.05$; $^{***}p < 0.001$ as determined by the Kruskal–Wallis test with Dunn's *post hoc* test. *P*-values of each fetal growth restriction (FGR) group are shown only in contrast to control group fetuses of the same sex. **(C)** Percentages show the distributions of males and females within the experimental groups. The FGR group contained the fewest males (25.8%) in comparison to the number of females (48.5%) but also contained the highest number of fetuses of unknown gender (25.7%), a finding that is mostly due to technical reasons because of the high degree of degraded DNA in the retarded cyanotic fetuses. In comparison, the control group contained 40.5% males, 52.4% females, and 7.1% fetuses of unknown gender; the doxycycline (Dox) control group contained 65.5% males, 32.8% females, and only 1.7% fetuses of unknown gender.

Figure S3 | Correlations of fetal weight and placental efficiency with either placental hsFLT1 transcript level **(A)** or fetal hsFLT1 transcript level **(B,C)** in the hsFLT1/rtTA mouse model. **(A)** Reduction in fetal body weight was negatively correlated with fetal expression of human soluble fms-like tyrosine kinase-1 (hsFLT1) ($r = -0.6691$; $p = 0.0033$). Placental efficiency exhibited a moderate negative correlation with the expression of fetal hsFLT1 ($r = -0.4059$; $p = 0.1188$) **(B)** and a weak negative correlation with the expression of placental hsFLT1 ($r = -0.2439$; $p = 0.24$) **(C)**.

Figure S4 | Analysis of the expression of important placental marker genes in the hsFLT1/rtTA mouse model. **(A)** Heat-map representation of the results of quantitative reverse transcription polymerase chain reaction (qRT-PCR) of 34 marker genes detected differences in placental gene expression between fetal growth restriction (FGR) homozygous ($n = 7$) or FGR heterozygous ($n = 15$) placentas and control ($n = 12$) or doxycycline (Dox) control ($n = 13$) placentas. The expression of human soluble fms-like tyrosine kinase-1 (hsFLT1) changed the expression of 22 of 34 genes, as shown by one-way analysis of variance (ANOVA; color-coding intensity in the red spectrum shows an increase in the expression of a marker gene, and color intensity in the blue spectrum shows a decrease in the expression of a given marker gene). **(B)** Protein levels of differentially regulated angiogenesis-related factors (Tissue Factor, Serpin E1, and Serpin F1) detected with Proteome Profiler Angiogenesis Antibody Array. All three factors were upregulated upon hsFLT1 expression in FGR hom ($n = 5$) and het ($n = 5$) placentas compared to control ($n = 5$) and Dox control ($n = 5$) placentas. Data is presented as mean ± standard deviation.

Figure S5 | Characterization of average DNA methylation of insulin-like growth factor two (*Igf2*) differentially methylated region two (*DMR2*) **(A)**, H19 imprinting control region (*H19-ICR*) **(B)** and global DNA methylation by long interspersed element one (*LINE1*) **(C)** in placentas of the human soluble fms-like tyrosine kinase reverse tetracycline-controlled transactivator (hsFLT1/rtTA) mouse model. Samples were obtained from complete placentas. Methylation levels were analyzed by pyrosequencing. The following experimental groups were analyzed: fetal growth restriction homozygous (FGR hom; $n = 4$); FGR heterozygous (het; $n = 7$); FGR wild-type (wt; $n = 7$) in various shades of red; and control ($n = 10$) and doxycycline (Dox) control ($n = 6$) groups in different shades of gray. Data are presented as means ± standard error of the mean. $^*p < 0.05$, as determined by the Kruskal–Wallis test with Dunn's *post hoc* test.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 26 March 2019
doi: 10.3389/fendo.2019.00179

Hormonal Changes Associated With Intra-Uterine Growth Restriction: Impact on the Developing Brain and Future Neurodevelopment

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The environment in which a fetus develops is not only important for its growth and maturation but also for its long-term postnatal health and neurodevelopment. Several hormones including glucocorticosteroids, estrogens and progesterone, insulin growth factor and thyroid hormones, carefully regulate the growth of the fetus and its metabolism during pregnancy by controlling the supply of nutrients crossing the placenta. In addition to fetal synthesis, hormones regulating fetal growth are also expressed and regulated in the placenta, and they play a key role in the vulnerability of the developing brain and its maturation. This review summarizes the current understanding and evidence regarding the involvement of hormonal dysregulation associated with intra-uterine growth restriction and its consequences on brain development.

Keywords: intra-uterine growth restriction, brain development, glucocorticoids, neurosteroids, insulin growth factor, thyroid hormones

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *26 November 2018*
Accepted: *04 March 2019*
Published: *26 March 2019*

Citation:
Baud O and Berkane N (2019) Hormonal Changes Associated With Intra-Uterine Growth Restriction: Impact on the Developing Brain and Future Neurodevelopment. Front. Endocrinol. 10:179. doi: 10.3389/fendo.2019.00179

FETAL GROWTH RESTRICTION, BRAIN DEVELOPMENT, AND HORMONES

Fetal growth depends on several factors of maternal, fetal and placental origin, in particular genetic background, nutrients and oxygen supply to the fetus, maternal nutrition and various growth factors and hormones (1). Suboptimal fetal growth is likely to be a key factor of disruption in brain development and many neurodevelopmental disorders of motor and cognitive dysfunction have their origins in the antenatal period (2, 3). Specifically, intra-uterine growth restriction (IUGR), defined as the inability of a fetus to reach its genetically determined size is closely linked to neurodevelopmental deficits. Indeed, infants exposed to IUGR conditions are at high risk not only for neonatal death and cerebral palsy (4), but also for other neurodevelopmental morbidities including mental retardation, a wide spectrum of learning disabilities and developmental behavioral disorders associated with the onset of neuropsychiatric disorders later in life (5–7). Several of these neurodevelopmental impairments are associated not only with deleterious effect of brain undergrowth but also with IUGR-related injury of the developing brain. Magnetic resonance imaging (MRI) has clearly revealed alterations of brain development in growth-restricted infants, involving both white and gray matter (8, 9) and including altered neural circuitry identified by diffusion MRI connectomics (10, 11) that correlate with functional cognitive, motor, and psychiatric deficits later in life (12, 13).

Hormonal balance has a crucial role in fetal growth and maturation, parturition, neonatal adaptation, and brain development (14). Hormones act as maturational and nutritional signals controlling tissue development and differentiation and closely interact with the *in utero* environment. Imbalance between hormones due to placental dysfunction or antenatal chronic stress conditions don't only impair fetal maturation and growth but could also induce obstetrical, perinatal and neonatal complications including cesarean section, perinatal asphyxia, respiratory distress syndrome, abnormal glycemic regulation or inappropriate adrenal function, and hypothalamus pituitary axis (HPA) responsiveness (5).

This review recapitulates state-of-the art data based on a search in the PubMed library in English for the key words "intra-uterine growth restriction," "hormones," and "brain development." The last search was done in October 2018. No restriction of year and authors were applied and review papers were used as references only for the general concepts. We identified 6 dysregulated hormones in case of IUGR, as a cause or as a result, closely related to brain development and future neurobehavioral outcomes, including glucocorticoids and oxytocin, estrogens and progesterone, insulin growth factor, and thyroid hormones.

FETAL GROWTH RESTRICTION AND CHRONIC EXPOSURE TO ENDOGENOUS GLUCOCORTICOSTEROIDS

Fetal Growth Restriction, Chronic Antenatal Stress, and Glucocorticoid Exposure

Glucocorticoids (GCs) are key mediators of stress responses involved during fetal development in the regulation of fetal growth and maturation of fetal tissues and organs (15, 16). Experimental and clinical evidence indicates that increased exposure of the fetus to GCs is associated with adverse outcomes including IUGR (17), postnatal hypertension, and cardiovascular disease (18, 19), postnatal glucose intolerance, increased postnatal activity in the HPA axis (20), and interference with fetal brain development (21, 22). Conversely, placental vascular diseases leading to IUGR were found to be associated with higher plasma cortisol and lower ACTH levels compared to eutrophic fetuses (23).

Besides high concentrations of GCs observed in pregnancies complicated by IUGR, their biological effects are dependent on glucocorticoid receptors (GR), mineralocorticoid receptors, and 11beta-hydroxysteroid dehydrogenase 1/2 (11β –HSD1/2) whose expression varies over time during the antenatal period. Speirs et al. demonstrated critical periods of GC sensitivity related to changes in the expression of these molecules during antenatal development in the mouse (24). Using *in situ* hybridization they showed that GR mRNA levels were very low at embryonic day (E9.5) in the fetus but not in the placenta, and then variably rose during gestation in several tissues, including the central nervous system (CNS). In humans, both GR and the MR are highly expressed in the hippocampus from 24 weeks of gestation (25).

Fetal 11β-HSD1 mRNA expression, which could enhance GC levels locally was detected at low levels in a few brain regions, including the hippocampus only after E16.5 in mice (24). In sheep, a specific increase in the expression of 11β-HSD1 mRNA in growth-restricted fetuses in late gestation has been reported (26). In the rat, placental 11β-HSD2 is considered as a "barrier" to endogenous GCs and genetic mutations of this enzyme were found to be associated with low birthweights (27). In humans, the 11β-HSD2 gene mutation also produces IUGR which is associated with reduced placental activity of this enzyme also highly expressed in the developing brain (28). Altogether, these data, both in animals and in humans, strongly suggest that the effects of high circulating cortisol levels associated with IUGR could be potentiated by specific changes in gene expression involved in their biological response in many tissues including CNS.

Glucocorticoids, Developing Brain, and Microglia Phenotypes

In humans, GCs regulate several developmental processes in the CNS, including hippocampal neurogenesis with variable effects on proliferation of progenitor cells, neurogenesis and astrogliogenesis in response to either low or high concentrations of cortisol (29). Low cortisol was found to increase proliferation and differentiation of progenitors into S100beta-positive astrocytes, and decrease neurogenesis. High cortisol was found to decrease proliferation and differentiation into neuronal cells without regulating astrogliogenesis. Inappropriate exposure to high levels of GCs early in pregnancy could therefore interfere with overall brain maturation. This programming effect of endogenous GCs affecting notably the HPA axis has been related to gene methylation and histone modifications associated with IUGR (30, 31) and can lead to long-lasting effects on the developing brain (32). IUGR also has sex-specific, persistent effects on hippocampal GR expression and its variants, a mechanism involved in HPA axis reprogramming, mostly in males (30).

GCs confer anti-inflammatory and immunosuppressive effects but are also able to potentiate, at high concentrations, inflammatory responses both at central and peripheral levels (33). In a model of restraint prenatal stress associated with higher levels of corticosterone investigated in juvenile and adult rats, a shift of the immune response toward a pro-inflammatory phenotype has been observed in adult rats (34). The change of GC receptor expression or function induced by IUGR could also change the microglia response toward pro-inflammatory insults associated with intensive care of growth-restricted infants, according to a multiple hit concept (35).

Extensive literature has demonstrated that chronic stress and GC exposure can impair the developing brain facing a large variety of insults, including hypoxia-ischemia, hypoglycemia, oxygen radical accumulation, all conditions potentially observed associated to IUGR (36). In preclinical models, IUGR-associated brain damage is usually associated with neuro-inflammation (5, 37–39), a key feature related to exacerbated activation of microglia, the resident macrophages of the CNS, able to

sensitize the developing brain to a secondary insult (40, 41). Microglial cells can acquire distinct phenotypes in response to perinatal stimuli that allow them to either disrupt developmental processes, i.e., myelination, synaptic pruning or axonal growth, or support repair, and regeneration. These diverse roles make microglia critical modulators of brain injury and GC exposure which are able to modulate microglial phenotype both in the developing and mature brain (42–45). In the developing brain, Gómez-González et al. showed that exposure to prenatal stress alters microglia maturation leading to an imbalance between immature and ramified microglia 1 day after birth in rat (46), and increased microglial activation in the hippocampus in juvenile animals (47, 48).

Balance Between GCs and Oxytocin

Oxytocin (OXT), an essential hormone during the perinatal period and parturition, is a neuropeptide released by the paraventricular nucleus and by the supraoptic nucleus of the hypothalamus, which is also known to be balanced against GCs. Indeed, studies carried out in rodents and in humans showed a close link between HPA axis activity and OXT release. OXT is also implicated in autism (49–51) and in the down-regulation of the central inflammatory response to injury in the mature brain (52, 53). In the developing brain, an association between IUGR, low expression of OXT and neuroinflammation, leading to defective myelination and abnormal brain function has been recently reported (54). Pharmacological treatment using carbetocin, a brain permeable long-lasting OXT receptor (OXTR) agonist, was found to be associated with a significant reduction of microglial activation and provided long-term neuroprotection. OTX also alleviated the HPA axis activation reducing GC release (55, 56), supporting the hypothesis of indirect anti-inflammatory action of OXT. These findings make OXT a promising candidate for neuroprotection, in particular in the context of IUGR.

SEX STEROID HORMONES

Sex Steroids in Human Pregnancy and Placenta

Estradiol (E2) and progesterone (P4) are highly expressed during pregnancy (57). Sex steroids are excreted by syncitiotrophoblasts into the intervillous chambers, entering the maternal circulation, and also the fetal vessels after crossing layers of cytotrophoblasts and stromal cells. While fetal circulating E2 and P4 are mainly of placental origin, hormonal concentrations differ between maternal and fetal circulation (58), implying that some of these hormones are converted in the villi (59, 60). 17β-hydroxysteroid dehydrogenase-2 (HSD17β2) converts E2, testosterone and Δ4-androstenedione (Δ4-dione) into estrone (E1), P4, and 20α-dihydroprogesterone, respectively (61, 62). These conversions could be involved in a protective effect from excessive fetal feminization or virilization, but could also play other roles (63). In primary culture of rat hippocampal neurons, it has been shown that E2 confers protection against excitotoxic-induced cell death (64), and some E2 metabolites have various effects on fetal brain development through their receptors by promoting neurite outgrowth, myelination, and synaptogenesis (3, 65, 66) as well as neuroprotective roles (67). P4 is already used as a treatment in human adult traumatic brain injury (68).

Interestingly, recent human studies have suggested that maternal serum concentrations of E2, P4 and some of their metabolites are modified during human pregnancy complicated by preeclampsia and/or IUGR with lower placental aromatization and E2 levels and higher P4 inactivation (20α hydroxylation) (69–75).

While growing evidence demonstrates that steroidogenic enzymes are highly expressed in the CNS, below, we describe data suggesting that changes in P4 and E2 induced by IUGR could have an impact on fetal brain development and adaptation to hypoxic stress.

Progesterone and Allopregnanolone and the Fetal Brain

Both in rodents and in humans, a large variety of brain structures (including olfactory bulb, hypothalamus, striatum, hippocampus, cerebral cortex, and cerebellum) and cell types (including glial, Purkinje, and Schwann cells) synthetize P4 (76, 77). Its 3α,5α-tetrahydroprogesterone (allopregnanolone) metabolite is mainly expressed in the cerebellum of neonatal rats (78). Progesterone receptors are expressed in rats by Purkinje cells and in the cerebellum.

Both P4 and allopregnanolone have a recognized role in neuroprotection (79). P4 has been found to induce inhibition of voltage-gated calcium channels (80) in the rat brain. Allopregnanolone found at high concentrations in maternal (81) and fetal sheep circulation (82) has been shown to have neurotrophic effects on neurons and glial cells (83, 84) in the ovine fetal brain (85).

In humans, available studies comparing controls and preeclamptic women reported conflicting results with unchanged, or higher maternal P4 concentrations (69, 74, 75). Using a reliable gas chromatography/mass spectrometry technique, no difference in maternal blood P4 and allopregnanolone concentrations was reported in women with vascular IUGR with or without preeclampsia compared to normal pregnancy (69). In contrast, in pregnant women with preexisting chronic hypertension, the development of preeclampsia was associated with higher allopregenalone concentrations (75).

In a model of IUGR developed in guinea pigs subjected to partial devascularization of the uterine horns during pregnancy, decreased allopregnanolone concentrations have been observed in fetal plasma and brain (79, 86). Moreover, in this model, despite increased expression of progesterone receptors in the brain, myelination was found to be decreased in the hippocampal region (87). Nevertheless, further studies are needed to better assess changes in P4 and its metabolite concentrations and signaling pathways involved in the adaptive response of the fetal brain to stress.

Estradiol

In rats, estradiol (E2) is synthetized by the hippocampus (88) and cerebellum (89) and potentially by other parts of the brain with some gender differences (90). E2 effects on the

brain are not yet well understood as both protecting (64, 91) and damaging effects (90, 92) have been described in primary cultures of rat hippocampal neurons. This hormone has been shown to promote axonal growth notably in cell cultures of fetal rat neurons derived from the ventromedial nucleus of the hypothalamus (93). In the oligodendroglial lineage, E2 also promotes the proliferation of immature oligodendrocytes, their differentiation into myelinating oligodendrocytes, and strongly reduces apoptotic cell death and neuro-inflammation in response to insult (94). On the other hand, as a potent regulator of the depolarizing actions of GABA, E2 can insult fetal brains subjected to hypoxic conditions by increasing the response to excessive GABA release via excess of free intracellular calcium (90).

Aromatase is a key enzyme for estrogen synthesis and several studies suggested a placental aromatase reduced activity in pregnancy with preeclampsia (70–75, 95) or with IUGR (69). As placenta is the major source of fetal estrogens, significantly lower maternal concentrations of E2, E1, or E1/Δ4-dione were reported in these pregnancies which may affect fetal estrogen levels. It can be hypothesized that during an IUGR pregnancy, a lack of fetal estrogens could disturb brain development. However, to our knowledge, no fetal or neonatal blood estrogen profile has been reported yet. Whether this abnormal placental steroidogenesis might induce changes in the steroid profile in the fetal compartment with potential brain insult remains to be determined. In addition, it is important to keep in mind that the developing brain itself has the capability to synthesize and convert sex steroids adding complexity to the interpretation of blood level data.

Despite these limitations, current evidence increasingly supports that E2, P4, and allopregnanolone play a key role in brain development and might be important modulators of brain vulnerability in the fetus with IUGR.

ROLE OF THE GLUCOSE-INSULIN-INSULIN-LIKE GROWTH FACTOR I (IGF-I) AXIS IN PLACENTAL AND FETAL GROWTH

Regulation of IGF Signaling in Growth Restricted Fetuses

The regulation of fetal growth depends not only on the nutrients available to the fetus but also on the regulation of Insulin-IGF/IGF binding protein 3 (IGFBP-3) axis (96, 97). The IGF factors I and II work together to control fetal growth through changes in size and function of the placenta. IGF-II is important for placental growth and development, and therefore allows more nutrients to reach the fetus. IGF-I acts as a "nutrient sensor" and finely regulates nutrient transfer across the placenta according to both the maternal environment and fetal demand. The production of IGF-I, particularly sensitive to maternal undernutrition and parental imprinting, regulates its signaling through its receptor (98). Disruption of this imprinting causes growth disorders including Beckwith–Wiedemann syndrome, associated with fetal overgrowth, and Silver-Russell syndrome, associated with IUGR (99). Several studies have shown that infants born growth restricted have lower levels of IGF-I, IGFBP-3, and insulin compared to appropriate for gestational age infants (100–102). The IGF system, IGF-I and IGF-II in particular, plays a critical role in fetal and placental growth. Disruption of the IGF-I, IGF-II, or IGF-IR gene induces IUGR, whereas disruption of IGF-IIR or overexpression of IGF-II enhances fetal growth (103).

Placenta and IGF Signaling

Many metabolic adaptations of pregnancy are regulated by placental hormones which undergo dramatic changes during gestation including placental estrogen and progesterone (104). Placental hormone expression is supposed to interact with fetal growth through polymorphic or epigenetic regulation of placental growth hormone (PGH) and human chorionic somatomammotropin (CSH) expression could alter the expression of other critical hormones including insulin or IGF-I (105). However, definitive evidence supporting that specific placental hormones are required for normal pregnancy and fetal growth is currently lacking. It is possible that other hormones of maternal origin, such as pituitary GH and/or prolactin, might partially compensate for reduced expression of placental hormones.

Defective IGF Signaling and Neurodevelopment

Abnormal fetal growth could also be associated with medically-induced preterm delivery (106). Low IGF-1 levels in very preterm infants and IUGR neonates were reported to be associated with high risk factor for adverse outcomes including chronic lung disease and retinopathy of prematurity. IGF-1 also plays crucial roles in the development and maturation (107) of the CNS with potent effects on cellular neuroplasticity, learning and memory, and confers neuroprotection following brain injury. IGF-1 acts at several sites to induce cellular plasticity through its receptor IGF-1R in neuronal and non-neuronal cells. IGF-1R is known to induce cellular plasticity by acting on glutamate receptors including AMPA/kaïnate-R, NMDA-R, calcium channels, and neurotransmitter release (108). Abnormal excitatory synaptic transmission observed in genetic diseases associated with behavioral disorders can be corrected by restoring SHANK3 expression or by treating neurons with IGF-1 (109). Regarding microglial activation, a major factor of brain injury, aging-related decrease in IGF-1 may contribute to the defective switch of microglia toward immunomodulatory and repair phenotype (110). During development, the IGF-1 level in cerebral spinal fluid is high, consistently with its important role in brain development, neuronal growth promotion, cellular proliferation, and differentiation (111). Finally, IGF-1 induces anti-inflammatory properties both in the developing and mature brain related to down-regulation of brain cytokine expression (112–114). Studies investigating the effects of intra-nasal IGF-1 demonstrated neuroprotection in models of LPS-induced white matter injury in the developing rat brain (115), cerebral hypoxic-ischemic injury (116, 117), and other neurodegenerative damages (118), probably through the phophatidylinositol-3 kinase/Akt pathway (119). However, the causality between low IGF-1

levels and neuroinflammation associated with IUGR remains to be confirmed.

THYROID HORMONES

Thyroid hormones are essential for fetal brain development and maturation. Severe but also mild or subclinical neonatal hypothyroidism has been associated with neurodevelopmental impairment (120–124). However, all neonates with subclinical or mild hypothyroidism are not identified by newborn screening programs (Guthrie test). Factors associated with neonatal hypothyroidism include prematurity and IUGR (125, 126). IUGR and/or preeclampsia can be the consequences of placental insufficiency associated with overexpression of sFlt-1 (soluble fms-like tyrosine kinase-1) a soluble form of the vascular endothelial growth factor- type 1 (VEGFR-1) (127), several weeks before the beginning of maternal clinical signs (128). sFlt-1 has anti-mitogenic properties on endothelial cells (129) by trapping VEGF and placental growth factor (PlGF) leading to hypertension and proteinuria (127, 130). When IUGR occurs in human, higher sFlt-1 or sFlt-1/PlGF ratio concentrations in maternal blood have also been observed compared to the control group (131, 132). A nested case control study showed that preeclampsia predisposes to reduced maternal thyroid function (transient or permanent) (133) as others report (134). This thyroid insufficiency seems to be mediated by sFlt-1 which impairs fenestrated capillary endothelium present in endocrine glands (135). By disrupting VEGF/VEGF-R signaling in adult mice, Kamba et al. showed capillary regression in different organs, the amount of regression was dose- and organ-dependent with the highest effect in thyroid (135). Recovery of thyroid capillary density has been observed within 2 weeks after cessation of treatment. Since sFlt-1 crosses the placenta, an impact of fetal thyroid function could also be suspected with risk of subsequent neurodevelopmental impairment. Cord blood sFlt1 concentrations have been found to be inversely correlated to birthweight (136) and free T4 and positively correlated with thyroid stimulating hormone (TSH) (137). However, conflicting findings have been reported regarding the effect of IUGR on fetal serum concentration of thyroid hormones. In a series of 49 growth-restricted fetuses who had cordocentesis during pregnancy, higher concentrations of TSH and lower concentrations of free T4 have been found compared to fetuses with appropriate growth for gestational age, and changes in TSH concentrations were correlated to fetal hypoxia and academia (138). In contrast, others reported unchanged or low TSH levels in IUGR cord blood compared to controls (139–142). Variability in fetal exposure to sFlt1 could be involved in TSH regulation and may partly explain these conflicting findings.

In summary, IUGR leads to neuropathological consequences for the developing brain with heterogeneous features and long-term neurocognitive and behavioral consequences (5, 143). Many factors contribute to the vulnerability of the developing brain, including age of delivery, severity of *in utero* compromise, co-morbidities occurring during the perinatal period, complications associated with medically-induced preterm delivery. Changes in several hormones strongly involved in the regulation of brain development and maturation are likely to play a key role. More research is needed to better understand these crosstalks.

AUTHOR CONTRIBUTIONS

OB and NB did the literature review and collectively analyzed articles selected in this review paper. OB wrote paragraphs related to introduction, glucocorticoids, oxytocin, and IGF1. NB wrote paragraphs related to sex steroids and thyroid hormones.

FUNDING

This work was supported by Fondation de l'Avenir.

ACKNOWLEDGMENTS

The authors thank Audrey Toulotte-Aebi for editing the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 28 March 2019
doi: 10.3389/fendo.2019.00186

School Age Neurological and Cognitive Outcomes of Fetal Growth Retardation or Small for Gestational Age Birth Weight

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Children who were growth restricted *in utero* (FGR) and are born small for gestational age (SGA) may experience poorer long term neurological and cognitive outcomes. Those also born preterm may have particular difficulties. The objective of this paper was to review the literature on school age neurocognitive outcome for term and preterm children that was published in the last 15 years. Considering term born children first, there is evidence that these children are at higher risk for Cerebral Palsy (CP) than those born appropriate for gestational age (AGA); information on neuromotor function in the absence of CP is somewhat contradictory. With regards to cognitive outcome, the most common finding was that being born SGA and/or FGR at term does not impact negatively on general intellectual functioning, commonly assessed by IQ scores. There was some indication that they may experience particular problems with attention. With regards to children born preterm, the risk of CP appears not to be increased compared to those preterms born AGA. For preterm children who do not develop CP, motor outcome is more affected by post-natal and post-neonatal brain growth than intrauterine growth. In contrast to term born children, preterm SGA and/or FGR children are at increased risk of cognitive and behavioral difficulties, and in common with term born children, are at higher risk than their AGA counterparts of difficulties with attentional control. In conclusion, preterm born SGA and/or FGR children are at higher risk of neurodevelopmental problems in the school years. It is important to continue to follow up children into the school age years because these difficulties may take time to emerge, and may be more visible in the more demanding school environment.

Keywords: small for gestational age, fetal growth restriction, cognitive outcome, neuromotor outcome, school age

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *20 November 2018*
Accepted: *06 March 2019*
Published: *28 March 2019*

Citation:
Vollmer B and Edmonds CJ (2019) School Age Neurological and Cognitive Outcomes of Fetal Growth Retardation or Small for Gestational Age Birth Weight. Front. Endocrinol. 10:186. doi: 10.3389/fendo.2019.00186

Low birth weight and poor fetal growth affects a significant proportion of newborns and pregnancies worldwide, and have been associated with a risk for impaired neurodevelopment across multiple domains (1, 2) for both individuals born preterm and born term, with a notion that outcomes for those born preterm are likely to be more complex than in those born term.

In the existing literature on neurodevelopmental outcomes, often, small for gestational age (SGA) and fetal growth restriction (FGR) are used interchangeably, but FGR is not the same as SGA.

This makes interpretation and comparison of outcome studies often complicated. Study-specific definitions of FGR, SGA and IUGR are included in the tables.

The definition of small-for gestational age (SGA) by the Royal College of Obstetrics and Gynecology (RCOG; https://www.rcog.org.uk/globalassets/documents/guidelines/gtg_31.pdf) in the UK, and also the American College of Obstetricians and Gynecologists, refers to a newborn with a weight or abdominal circumference at birth at less than the 10th centile, either according to population based growth charts or centiles that take into account factors such as gestational age, sex, ethnicity, and maternal characteristics. Further, this can be divided into normal (i.e., constitutionally small), non-placenta mediated growth restriction (for example, chromosomal abnormalities, syndromes, infections), and placental mediated growth restriction. Fetal growth restriction implies that the fetus cannot achieve its growth potential, and this is often indicated by abnormal Doppler studies. Over half of those who are SGA have appropriate fetal growth ("constitutional" SGA), although the likelihood of also having FGR is higher in SGA. Fetal growth restriction can be classified into symmetrical (both body weight and head circumference are affected) and asymmetrical (body weight and/or length may be affected, head size is normal) and according to the time of onset of FGR (early, i.e., before 28 weeks of gestation, late, i.e., after 28 weeks of gestation) (3). Time of onset of FGR is an important factor since early and late onset FGR are distinct phenotypes in terms of placental dysfunction and effects on the brain. Early and late onset FGR affect the brain at different developmental stages and it is therefore likely that different brain regions are affected in a different way, which may partly explain the different outcomes in these two groups (4). Fetal growth retardation and preterm birth are often associated (5). Preterm birth *per se* poses a risk for long term neurological and developmental impairment and the combination with FGR is likely to add to this risk, however, it may be difficult to disentangle the effects of FGR from the effect of prematurity, and, indeed there have been somewhat inconsistent and contradictory finding in existing studies.

Studies have reported differences for the above described groups in neurodevelopmental outcomes, generally reporting favorable outcomes for those with constitutional SGA, whereas those with placenta-mediated FGR appear to be at risk for cognitive and/or behavioral problems later in life (6). In addition, within the FGR group, differences in outcomes are reported between those with symmetrical and those with asymmetrical FGR (7), although, again, some contradictory study results exist. Overall, existing studies are heterogeneous, for example, with regards to definition of growth restriction and small for gestational age, time of onset of FGR, inclusion and exclusion criteria, outcome measures, all of which makes comparison between studies difficult.

Nevertheless, some common themes emerge and in this narrative literature review we will summarize evidence on neuromotor and cognitive long term outcomes of SGA at birth and after FGR. The focus will be on school age outcomes rather than earlier outcomes as toddler age or adult age outcomes, and this will be described separately for children born at term age and for those born preterm. We considered literature published in the last 15 years. We have excluded studies that included children with underlying chromosomal abnormalities or teratogenic exposure.

MEASURES USED IN STUDIES ON SGA/FGR OUTCOMES

While the use of standardized assessments makes cross-study comparison possible, some of the assessments used were more individual and depended on the study location and specific sample. For example, some studies used information from military conscription (8), national registers of learning disability (9), and/or and information extracted from medical records (10).

Neuromotor and neurosensory outcomes are described in some, but not all, studies. In the studies that did describe neuromotor outcomes, only seldom a standardized approach was applied for neurological examination. Diagnosis of Cerebral Palsy (CP) was either based on direct neurological examination assessing posture, movements, muscle tone, and reflexes, or on information extracted from medical notes. In a few studies, and when diagnosis was based on examination, the Surveillance of Cerebral Palsy in Europe (SCPE) criteria for CP were used (11). Functional impairment in those with CP was not always defined, but in some studies the Gross Motor Function Classification System (GMFCS, (12) was used for overall motor function classification. In one study, independently of a diagnosis of CP, gross motor function was assessed using the GMFCS, and hand function using the Manual Ability Classification System (13). The most commonly used tool for assessment of motor skills was the Movement ABC for Children (14), a standardized tool, which assesses dexterity, balance skills, and ball skills. Another standardized test, the Peabody Developmental Motor Scales (15), testing reflexes, stationary, locomotion, object manipulation, grasping, and visual-motor integration, has also been used.

Very rarely are visual function and hearing function described specifically, and, if so, this information was mainly extracted from medical records and reported as a binary outcome (impairment/no impairment).

Abbreviations: AGA, appropriate for gestational age; ANT, Amsterdam Neuropsychological Tasks; AGR, asymmetric growth restriction; ARED, absent or reversed end-diastolic blood flow; BW, Birth weight; BL, Birth Length; BRIEF, Behavior Rating Inventory of Executive Function; CLD, Chronic lung disease; CP, Cerebral Palsy; FGR, Fetal growth restriction; FSIQ, Full Scale IQ Score; GA, Gestational age; HC, Head Circumference; HGR, Head growth restriction; ICH, Intracranial hemorrhage; IQ, Intelligence Quotient Score; IUGR, Intrauterine growth restriction; K-ABC, Kaufmann Assessment Battery for Children; M-ABC, Movement ABC for Children; MHPT-Moray House Picture Test; NEPSY, A Developmental Neuropsychological Assessment; PIQ, Performance IQ Score; POBW, Percentage of optimal birth weight; RAVLT, Rey's Auditory Verbal Learning Test; ROYCF-Rey Osterreith Complex Figure test; SES, Socio-economic status; SGA, Small for gestational age; TeaCH, Test of Everyday Attention in Children; VIQ, Verbal IQ score; WASI, Wechsler Abbreviated Scales of Intelligence; w, Week; WGR, Weight growth restriction; WISC, Wechsler Intelligence Scales for Children; WISC-R, Wechsler Intelligence Scale for Children – revised; WPPSI, Wechsler Preschool and Primary Scale of Intelligence; WRAT, Wide Range Achievement Test; y, Year.

For outcome measures used to assess general cognitive abilities and specific cognitive functions, there was a large and heterogeneous range of assessments reported, but most of the outcome measures used were standardized neuropsychological tests and questionnaires. For assessing general intellectual functioning, the most frequently used assessment was the Wechsler Intelligence Scale set, most commonly the Wechsler Intelligence Scales for Children (WISC) (16), but also the Wechsler Abbreviated Scales of Intelligence (WASI) (17), and the Wechsler Preschool and Primary Scale of Intelligence (WPPSI) (18). The Wechsler Scales assess performance across cognitive domains and usually include an overall IQ score, and performance and verbal IQ scores. General intellectual functioning was also assessed using the Kaufman ABC (19). More focused neuropsychological assessments were used to examine specific aspects of performance, including attention (the Test of Everyday Attention in Children—TEA-Ch) (20), memory (Rey's Auditory Verbal Learning Test—RAVALT) (21), and design copying of the Rey Ostereith figure (22). Executive functioning was assessed using the NEPSY (23), and parental ratings of children's behavior were also employed. Children's executive functioning at home and/or at school were assessed using the Behavior Rating Inventory of Executive Function (BRIEF) (24). The Strengths and Difficulties Questionnaire (SDQ) (25), a brief behavior screening questionnaire, has parents and/or teachers rate children on emotional symptoms, conduct problems, hyperactivity/inattention, peer relationship problems, and prosocial behavior.

The development of typical and atypical social behavior was assessed using the Social Responsiveness Scale (SRS) (26), and screeners for autism included the Social Communication Questionnaire (SCQ) (27) and Autism Diagnostic Observation Schedule (ADOS) (28).

SCHOOL AGE OUTCOMES OF SGA AND/OR FGR IN CHILDREN BORN AT TERM

The study of neurocognitive outcome after fetal growth restriction in term infants is not subject to the same confounding factors as that in preterm infants because many of the mechanisms that affect preterm birth do not affect term infants. Nevertheless, with studies using different indicators of growth restriction, such as strict SGA criteria or birth weight as a continuum, it can be difficult to make cross study comparisons. **Table 1** reports study details from studies that examined school age neurocognitive outcome of SGA and/or FGR in the past 15 years. Of these, most studies defined SGA as birth weight <10th centile (9, 10, 32, 37–40, 46, 48, 49). Two studies assessed growth in head circumference from the prenatal period and/or birth to later childhood (34, 36). Three studies defined FGR in relation to birth weight standard deviation scores (8, 44, 48). Thus, there is disparity with the definition of SGA and FGR across studies that could affect the results. Only 2 studies included information on placental insufficiency, which would allow FGR to be identified rather than babies being SGA (38, 40). Furthermore, some of the studies of term born children also include a proportion of preterm babies (8, 40, 44).

Neuromotor Outcomes

Studies assessing only children with CP are reported here and not included in the table (10, 48, 49). An association between Cerebral Palsy (CP) and having been born SGA and/or FGR has been reported for children born at term in outcome studies on SGA and/or FGR, and data from Cerebral Palsy registers support this association (11, 50, 51). Whether FGR leads to CP or whether abnormal brain development that causes CP leads to FGR is debated.

The Surveillance of Cerebral Palsy in Europe (11) group report an increased risk of CP, across the gestational age range, for those born below the 10th centile and above the 97th centile [using the North of England standard (52) and fetal growth standard by Maršál et al. (53)]. Data from the Canadian Cerebral Palsy Registry (10) and from other reports based on CP registry data (49, 50, 54) show that those with CP who were born term and SGA compared to those with CP born AGA had more frequently intrauterine infections, small head circumference at birth, maternal gestational hypertension, placental abnormalities, perinatal asphyxia, and delivery by emergency cesarean section, amongst other risk factors, such as birth defects (congenital microcephaly, teratogenic, genetic, and syndromal) as reported by Blair et al. (48), for the Australian CP register cohort. This illustrates that it has to be kept in mind when interpreting findings on associations between SGA and/or FGR with CP that SGA populations in CP registers tend to include a heterogeneous group of SGA children, i.e., also children who had other risk factors for SGA and/or FGR than placenta-mediated FGR.

Many of the prospective follow-up studies examining neurodevelopmental outcomes of SGA and/or FGR at term excluded children with CP or other co-morbidities, and of those that did not exclude them, few reported on the presence or absence of CP, or neurological signs in the absence of CP. Leitner et al. (40) compared the occurrence of neurological signs in the absence of CP in children who were born with BW <10th centile with a group of children born AGA at age 9 years; all SGA children had onset of FGR in the mid-second to third trimester, verified by fetal ultrasound, and all had asymmetric FGR. They found a significant difference in the quality of neurology between those born after FGR and those born AGA, with poorer scores for to motor coordination, timed coordination performance, grapho-motor skills and lower muscle tone in the FGR group. Similarly, Emond et al. (31), who used the M-ABC at age 8 years found, after controlling for socio-economic variables, significantly lower scores for dynamic balance and eye–hand coordination in those born SGA (<10th BW centile) but similar performance to those born AGA for manual dexterity. Tanis et al. (44), in contrast, did not find a significant difference between a group of children born at term and SGA and a group born AGA at the age of 7 years when assessed with the M-BAC (dexterity, balance, ball skills) and on tests of visuo-motor integration. The findings by Tanis et al are consistent with those by Sommerfelt et al. (42), in 5 years old children born <15th centile (Norwegian Birth Registry standards), who did not find significantly poorer

TABLE 1 | Included studies for term born children.

References	Study type	Definition of fetal growth	Participants	Neurology/Neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Beukers (29)	Prospective follow up study comparing term FGR and term AGA children	BWt ratio <10th centile; BWR calculated as BW/expected weight for GA, using the Gardosi customized fetal growth chart 50th centile values (30)	96 term children with FGR; 32 term and birthweight ≥ 2,500 g BY 200–2003 Age at assessment: 12 y	Not assessed	*Child assessment:* IQ–Wechsler Intelligence Scale for Children (WISC). Executive Functioning– Impulse control (stop task); Verbal working memory (WISC Digit span); Visual working memory (Spatial Temporal Span of the Amsterdam Neuropsychological Tasks–ANT); set shifting (Shifting visual set–ANT); planning (Tower of London). *Parent ratings:*- Behavior Rating Inventory of Executive Functions Dutch version (BRIEF)	No difference in IQ, executive functioning, attention or parent rating of EF between FGR and controls. Parent report FGR children more social and attention problems	Neurocognitive outcomes including IQ, executive function, memory and attention comparable in FGR children AGA controls Parent ratings suggested elevated levels of social and attention problems
Emond (31)	Population based cohort, north east Brazil	Low BW: BW 1,500–2,499 g ($n = 202$) Appropriate BW: BW 3,000–3,499 g ($n = 212$); matched for sex and month of birth	Born ≥37 wGA BY 1993–1994 Age at assessment: 8 y 40% of original cohort assessed ($n = 83$ low birth weight; $n = 81$ appropriate birth weight) Analyses adjusted for socio-economic background variables Additional analyses performed to examine head size at birth, age 6 m and 8 y with IQ at age 8 y	Neurological status (muscle tone, focal neurological signs) M-ABC for neuromotor function *Findings:* No significant difference in frequency of focal neurological signs	WISC-III for Full Scale IQ, Performance IQ, Verbal IQ Memory (auditory and verbal) assessed with WISC-III subtests TeaCH for attention abilities SDQ for behavior	After controlling for socio-economic background no significant difference between BW groups for IQ scores, memory function Significant differences between BW groups for dynamic balance skills and eye-hand coordination, selective attention; peer relationships Head size at birth, age 6 m and 8 y significantly associated with IQ age 8 y	Low birth weight not associated with general cognitive abilities, except selective attention. Neuromotor function poorer in low birth weight children. Post-natal head growth more important than birth weight.
Fattal-Valevski (32)	Prospective follow-up study	BW <10th centile for GA according to Israeli birth weight curves (33); Growth data (weight, height, head circumference) from 1 to 2 y, 2–6 y, and 6–9 y	136 IUGR children BY 1992–2002 Age at assessment: Annual follow-up from birth; current assessment 9–10 y	Neurodevelopmental examination score including soft neurological signs. Score expressed as percentage of optimal items out of the total.	Short term memory, coordination skills; IQ assessed by the Wechsler Intelligence Scale for Children—revised (WISC-R)	BW, BL and GA positively correlated with 9–10 y neurodevelopmental score BW, BL, but not GA, positively correlated with 9–10 y IQ scores Better outcome for those with asymmetric IUGR than symmetric	Correlation of IQ with BW, BL but not GA suggests that there is better outcome for IUGR children delivered early Correlation of GA with neurodevelopmental score, suggests prematurity affects other areas of development

(Continued)

TABLE 1 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/Neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Gale (34)	Prospective cohort study	HC growth–expressed as Standard Deviation Score (SDS), measured at 18 weeks gestation and birth, and 9 y, using the British 1990 growth reference data (35)	221 children assessed at 18 weeks gestation, births, 9 months and 9 years BY 1992–1994 Age at assessment: 9 y	Not assessed	Wechsler Abbreviated Scales of Intelligence (WASI)—Full Scale IQ (FSIQ), Verbal IQ (VIQ) and Performance IQ (PIQ)	No significant associations between head circumference at 18 weeks gestation or birth and 9 years old IQ scores. Post-natal head group was associated with IQ. After adjusting for sex, FSIQ, VIQ and PIQ increased with each SD increase in head circumference up to 9 months, and to 9 years. The highest FSIQ were observed in children who had large increases in HC between birth and 9 months, and a further increase between 9 months and 9 years. This was not linked to maternal or home characteristics other than maternal education	Post-natal brain growth is more important than prenatal brain growth for IQ Post-natal head growth greater in children of mothers educated to degree level, or of higher SES
Gale (36)	Prospective cohort study – the Avon Longitudinal Study of Parents and Children cohort (ALSPAC). The cohort studied is the Children in Focus cohort – a random 10% selected from ALSPAC.	HC growth - measured at birth, 4, 8, 12, 25, 31, 37, 43, 49, 61, and 96 months. HC in analyses –birth, 1, 4, and 8 years—expressed as z scores obtained from the study group. Growth between time points calculated	633 children BY 1991–1992 Age at assessment: 8 y	Not assessed	IQ assessed by Wechsler Intelligence Scale for Children (WISC) at 8 years	Children with larger HC had higher IQ scores For each 1 SD increase in HC– after adjusting for gender, GA, parental factors—FSIQ, VIQ and PIQ increased age 8 years IQ at 8 years was associated with HG during infancy	Above average head growth during infancy associated with above average IQ scores at 8 years Post-natal brain growth during infancy more important to later IQ than growth at other times Brain growth after infancy may not compensate for slower growth in the first year of life
Geva (37)	Population based prospective follow up study of single center (Tel Aviv, Israel)	BW <10th centile for GA according to Israeli birth weight curves (33), those with genetic disorders and unrelated co-morbidities excluded	110 IUGR children born at term, 63 control AGA children BY 1992–1995 Age at assessment: 9 y	Not assessed	Visual aural digit span test for digit span—aural-oral, visual-oral, aural-written, visual-written; RAVLT (superspan list learning test); Rey Osterreith Complex Figure test (ROYCF)	Learning functions over trials were similar for IUGR and controls on RAVLT. IUGR children performed more poorly than controls on ROYCF. IUGR children worse performance on immediate digit span than controls. Performance on digit span output mode did not differ between IUGR and control. IUGR children have memory problems particularly when the input is aural.	Memory difficulties in children born IUGR in the short term, not observed in later stages of learning (i.e., processing, consolidation and retrieval not affected) Difficulties when recalling both verbal and complex visuo-spatial material

(Continued)

TABLE 1 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/Neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Geva (38)	Population based prospective follow up study of single center (Tel Aviv, Israel)	BW <10th centile for GA according to Israeli birth weight curves (33), those with genetic disorders and unrelated co-morbidities excluded	138 IUGR children born at term, 64 control AGA children BY 1992–1995 Age at assessment: 9 y	Not assessed	VADS Digit span–aural-oral, visual-oral, aural-written, visual-written; IQ assessed by Wechsler Intelligence Scale for Children–revised (WISC-R)	IQ significantly lower in IUGR group. IUGR children performed worse than controls on verbal STM tasks.	Short term memory difficulties in IUGR children are particularly observed when material presented aurally and oral response required General cognitive performance also poorer in IUGR children relative to controls
Lawlor (39)	Single center, population based prospective birth cohort -Aberdeen Children of the 1950s cohort.	IUG rate measured as BW standardized for gender and GA. SGA calculated as BW < 10th centile, using a customized birthweight-for-gestation standard for the whole cohort	9,792 singleton children and 1,645 sibling pairs BY 1950–1956 Age at assessment: 7, 9 and 11 y	Not assessed	General ability measured by the Moray House picture test (MHPT)	Positive linear association between BW and MHPT, even when adjusted for SES, Maternal height and age, gender and GA. Within sibling comparison of SGA vs. AGA showed no difference in MHPT scores. If AGE compared to SGA outside of sibling pairs, AGA performed more poorly	While BW correlates with general cognitive ability within-sibling analyses suggest that associations between AGA status and cognitive ability in singletons is explained by within family factors, including SES, parental education and intelligence, genetic factors and fixed maternal factors
Leitner (40)	Prospective birth cohort	BW < 10th centile for gestational age, according to the Israeli percentile curves published by Lieberman et al. (33). Late onset IUGR (mid 2nd to 3rd trimester; verified by fetal ultrasound)	123 children with IUGR and 63 AGA controls, 30% of the sample were preterm BY 1992–2002 Age at assessment: 9–10 y Children with CP or severe neurological deficits were excluded	Neurological examination from birth, follow-up and at 9–10 years. 72 item examination. At age 9 years assessment of co-ordination skills, tone, timed performance, graphomotor skills. *Findings:* IUGR group poorer performance in coordination, timed performance, graphomotor skills; lower muscle tone	IQ assessed by Wechsler Intelligence Scale for Children (WISC), School achievement by Kauffman Assessment Battery for Children (K-ABC)	IUGR children performed more poorly than AGA controls on IQ and school achievement, where learning difficulties, particularly language-based difficulties were observed. Attention span, speech and language abilities were poorer in the IUGR group. Subgroup analyses compared IUGR children whose height, weight and head circumference were above ("optimal") or below ("suboptimal") the 10th growth percentile at age 9–10 years–the "suboptimal" catch up group performed worse in neurodevelopmental and cognitive tests than "optimal" group	Children with IUGR perform more poorly than their AGA peers on tests of IQ, school achievement, and neurological examination. Early growth ameliorates these group differences

(Continued)

TABLE 1 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/Neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Leonard (9)	Population-based record linkage study of children in Western Australia born 1983–1992 with intellectual disability of unknown cause	Percentage of optimal birthweight (POBW) categorized as %–POBW <85% was equivalent to <10th centile of optimal BW based on data from the 1998–2002 Western Australia birth cohort (35)	2,865 children with intellectual disability, including both term and preterm children BY 1983–1992	Not assessed	Registrations with the Disability Services Commission or educational services defined as "mild to moderate" (85.9%), "severe or profound" (7.4%), Autism spectrum disorder (6.7%)	In infants born at term, less than optimal fetal growth (POBW < 85%) was associated with "mild to moderate" intellectual disability, POBW < 75% was associated with "severe" intellectual disability. These were after adjusting for factors including SES and paternal occupation	Less than optimal uterine growth associated with intellectual disability in term born children. This persists after adjusting for sociodemographic factors. Severe growth restriction in term children associated with severe intellectual disability
Lundgren (8)	Population based cohort study of singleton male infants born from 1973 to 1976 and alive aged 18 years	"Light for gestational age" defined as > −2 SDs from mean BW for GA, AGA defined as between −2 and +2 SDS, "heavy for GA" defined as > +2 SDs from mean BW according to Swedish birth standards (41)	168,068 males conscripted for national service BW 1973–1976 Age at assessment: 18–25 y This study includes term and preterm adults	Not assessed	General intellectual performance assessed on four dimensions, logical/inductive, verbal, spatial and theoretical/technical	Being born SGA associated with increased risk of subnormal performance on all outcome measures compared to those born AGA SGA and small HC conferred additional risk of subnormal logical performance, SGA and short adult stature associated with a 40–50% increased risk in all outcome measures	Being born SGA associated with increased risk of impairment on logical/inductive, verbal, spatial and theoretical/technical dimensions of intellectual performance
Sommerfelt (42)	Multicenter study on causes and consequences of *in utero* growth retardation, the NICHD Study of Successive Small-for-Gestational Age Births (NSSSAB) 3 geographical Scandinavian regions; mothers recruited before 20 wGA	SGA = BW were <15th centile for gestation according to reference standards from the Norwegian Birth Registry (43); above 15th centile AGA	321 SGA and 312 AGA (78% of eligible children) Recruitment <20 w GA between January 1986 and March 1988 Age at assessment: 5 y	PDMS for balance, eye-hand coordination, locomotor abilities., Grooved Pegboard test for manual dexterity *Findings:* Motor problems more frequent in SGA group but no significance group difference	WPPSI for IQ scores (Verbal IQ, Performance IQ) Norwegian version of the Illinois Test of Psycholinguistic Abilities for receptive language and short-term memory	No significant difference in IQ scores, memory tests SGA with poorer motor performance lower scores on tests of visuospatial, manual dexterity, and verbal function	SGA group differs little from AGA peers on neuropsychologic profile and neuromotor outcome SGA group weakness in visuo-spatial skills and manual dexterity

(Continued)

TABLE 1 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/Neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Tanis (44)	Population based regional cohort—subgroup (Longitudinal Preterm Outcome Project study), Netherlands	BW < −1 SD and BW > −1 SD according to GA; Dutch Kloosterman curve (45). Cut-off for SGA (> 1 SD, below the 16th percentile) according to Etude Epidemiologique sur les Petits Ages Gestationnels study	42 SGA children (10 full term) and 336 AGA children (120 full term), Analyses combine MPT and term –GA range is 31–41. Both SGA and AGA groups consisted of mostly preterm children BY 2002–2003 Age at assessment: 7 y	Motor outcomes assessed by Movement Assessment Battery for Children (M-ABC) *Findings:* No significant difference in motor skills between AGA and SGA group	Full Scale IQ scores (FSIQ) from the Wechsler Intelligence Scale for Children (WISC). Selective attention and Attention control assessed by the Test of Everyday Attention in Children (TEA-Ch). Verbal memory assessed using Rey's Auditory Verbal Learning Test (RAVLT). Visuomotor integration assessed by Neuropsychological Assessment. Executive functioning in daily life assessed by the Behavior Rating Inventory of Executive Function (BRIEF)	No differences between AGA and SGA children on FSIQ, attention, verbal memory, visuomotor integration, executive functioning. SGA children 4 times more likely to be in the abnormal range on the Attention Control measure than AGA children	SGA and AGA children similar on IQ, verbal memory, executive functioning and movement tests, but SGA children more likely to be in the abnormal range on attention control
Theodore (46)	Case-control follow up study	SGA defined as BW < 10th centile for GA for New Zealand standards (47)	241 SGA children and 348 aGA controls BY 1995–1996 Age at assessment: 7 y	Not assessed	Full Scale IQ scores (FSIQ) from the Wechsler Intelligence Scale for Children (WISC)	No significant difference in FSIQ between SGA and AGA children. In the whole sample, parental education and birth order were associated with FSIQ	No long term negative effect of being born SGA on FSIQ scores

AGA, appropriate for gestational age; BW, birth weight; BL, Birth Length; BY-birth year; FGR, fetal growth restriction; GA, gestational age; HC, Head Circumference; IUGR, intrauterine growth restriction; POBW, Percentage of optimal birthweight; SES, socio-economic status; SGA, small for gestational age; w, week; y, year. ANT, Amsterdam Neuropsychological Tasks; CP, Cerebral Palsy; BRIEF, Behavior Rating Inventory of Executive Function; K-ABC, Kaufmann Assessment Battery for Children; M-ABC, Movement ABC for Children; MHPT-Moray House Picture Test; NEPSY, A Developmental NEuroPSYchological Assessment; RAVLT, Rey's Auditory Verbal Learning Test; ROYCF-Rey Osterreith Complex Figure test; PDMS, Peabody Developmental Motor Scales; SDQ, Strengths and Difficulties Questionnaire; STM, short term memory; TeaCH, Test of Everyday Attention in Children; VADS, Visual aural digit span test; WASI, Wechsler Abbreviated Scales of Intelligence; WISC, Wechsler Intelligence Scales for Children; WISC-R, Wechsler Intelligence Scale for Children – revised; WPPSI, Wechsler Preschool and Primary Scale of Intelligence; WRAT, Wide Range Achievement Test. IQ, Intelligence Quotient Score; FSIQ, Full Scale IQ Score; PIQ, Performance IQ Score; VIQ, Verbal IQ score. The terms SGA, IUGR, FGR were used as in the study that is described.

performance in the SGA group when compared to the AGA group on testing with the M-ABC.

Cognitive and Behavioral Outcomes

In the reviewed studies, many different measures of neurocognitive outcome were employed, which can lead to difficulties summarizing results across studies. With this in mind, the first outcome to be discussed is that which was most commonly used across the reviewed studies—general intellectual functioning. IQ scores acquired from the Wechsler tests were the most commonly reported general cognitive outcome measures (29, 31, 32, 34, 36, 40, 42, 44, 46). There was some cross-study consensus about the effect of fetal growth restriction at term on Wechsler derived IQ scores, with three studies reporting that term FGR children's IQ scores were no different from those of control children (29, 42, 44, 46). In contrast, one study reported that FGR children's IQ scores were significantly lower than controls (40), but it should be noted that this study included both term and preterm children and thus this finding may not be isolated to term FGR children. One study found significant positive correlations between IQ scores and birth weight (32). A further two studies used head circumference at birth as an indication of fetal growth restriction (34, 36) and found that, for optimal IQ scores in later childhood, post-natal head growth in term born children was more important than prenatal head growth (34). In addition, the amount of post-natal head growth in term born children was greater for children whose mothers had been educated to degree level, or whose families had higher SES, which could be a consequence of cognitive stimulation and could also be linked to better nutrition (34). General intellectual functioning was also assessed by standardized measures other than the Wechsler range of tests, including the Kauffman ABC (40) and the Moray House picture test (39). Term children with FGR showed weaker performance than controls on both these assessments. In addition, higher levels of learning difficulties were reported, particularly language-based difficulties, and these were reflected by parental report of increased incidence of special educational needs interventions, particularly remedial education, neurological follow up, speech therapy and psychological intervention (40). However, this sample included 30% preterm children. While scores on the Moray House picture test were not affected by AGA status, birth weight as a continuous variable was associated with scores (39).

Taken together, the most common finding is that being born SGA at term does not impact significantly negatively on general intellectual functioning. The majority of studies that specifically examine FGR term children report similar performance on IQ-type measures as those found in controls (29, 39, 44, 46). Two studies reported that FGR term children have lower general intellectual functioning than controls (39, 40), but one of these studies also included a minority of preterm children, whose presence could account for these results (40). The other of these two studies (39) reported on children with late onset IUGR (occurring in the second to third trimester), thus it is possible that differences in the timing of growth restriction across studies impacts on the reported outcome; as not all studies reported the time at which growth restriction occurred, this interpretation is speculative. Birth weight correlates with later IQ scores in some studies (32), but not all (31), but other studies have reported that this occurs even in the normal birth weight range and therefore is not specific to FGR children (55).

As well as assessing performance on tasks measuring general intellectual functioning, the reviewed studies considered the effect of being born SGA at term on a range of more specific cognitive processes. However, as there was little consensus in either the aspects of cognition examined or the tests used, it is not possible to draw strong conclusions about specific outcomes, but any trends in the literature are considered here. Three studies examined memory processes (29, 38, 44). There was no difference between FGR term children and AGA term controls on a standard, orally presented digit span test or visual working memory (29), or differences between SGA and AGA children on a verbal list learning task (RAVLT) (44), although it should be noted that this latter study included both preterm and term SGA children. When performance on a digit span test that systematically varied both the presentation and recall of numbers was examined, the performance of FGR children was characterized by particular difficulty when the presentation was aural and children were required to make an oral response (38). While these findings require corroboration, they could have implications for classroom teachers as they suggest it might be more useful for children with FGR ifs teaching materials were non-aural (e.g., written/picture material) and children's responses should not always require them to speak aloud, but perhaps make written responses instead.

Two studies explored whether term born SGA children experience difficulties with executive functions, which describes a range of cognitive functions whose neural correlate lies in the frontal lobes and are involved with cognitive control of behavior (56). Executive functions are assessed both by performance on neurodevelopmental assessments, and also by questionnaire based rating scales, often completed by parents, which given an indication of how poor executive control can affect children's daily lives. The evidence on the effects of FGR on term born children's executive functioning was limited and mixed. For example, no differences in performance between FRG and AGA controls were found on a measure of impulse control (stop task) (29), and no differences between term SGA or term AGA on the parent rating form the BRIEF (44). However, SGA children were four times more likely to be in the abnormal range on TEA-Ch Attention Control compared to AGA children (44), although it should be noted that this sample included children born at term or preterm. Even in term born children without growth restriction, head circumference at birth and head circumference to length ratio at birth were predictive of ADHD symptoms at age 5 to 6 years (57). Term born low birth weight children had particular problems with attention (31). Thus, there is limited evidence in terms of the number of studies and within these studies, there is no reliable pattern of results on executive functions in term born FGR children. There were other measures assessed in individual studies only, with no cross-study comparison possible. For example, the presence of communication problems identified from children's medical

charts did not differ between AGA and SGA term children who had CP (10).

Some of these studies of term born SGA children highlight the period of catch up growth that is particularly important for long term neurocognitive outcome. General intellectual functioning in childhood is influenced by early brain growth–indexed by growth in head circumference–that occurs in infancy. For example, IQ score at 8 years was associated with greater than expected head growth during infancy, rather than head circumference at birth (31, 36), catch up growth at 2 years predicted 9–10 years IQ scores (40). Later growth is also important for IQ; children with optimal somatic catch-up growth at age 9–10 years, but not 2 years (defined as >10th centile in height, weight and head circumference) had significantly higher scores on neurodevelopmental and cognitive tests at 9–10 years than children with suboptimal catch-up growth (defined as <10th centile in height, weight or head circumference) (40). However, there seem to be better outcomes for term born children with asymmetric FGR, which favors head growth over the rest of the body, compared to children with symmetric FGR (32); this suggests sparing of the head, a neuroadaptive modification to conserve the developing brain.

SCHOOL AGE OUTCOMES OF SGA AND/OR FGR IN THE CONTEXT OF PRETERM BIRTH

Neurodevelopmental outcome of SGA and/or FGR in preterm (born <37 w GA; very preterm = born <32 w GA; extremely preterm ≤28 wGA at birth) infants is difficult to study since there it is complicated separate the effects of SGA/FGR from the consequences of preterm birth *per se*. The majority of studies investigating school age outcomes of SGA or/and FGR in preterm born children have excluded children with congenital abnormalities, congenital infections, chromosomal abnormalities, or syndromes. However, only in some studies detailed information on antenatal growth is available, and seldom are SGA and FGR clearly distinguished and examined separately; some studies may include constitutionally "small at birth" children. Definitions of SGA and growth references used differ between studies. All this is likely to affect study findings and needs to be kept in mind when interpreting and comparing results from different studies.

Table 2 provides study details from 10 studies on neurocognitive outcomes in preterm school aged children, the majority of which are population based studies that examined outcomes of FGR or SGA published in the past 15 years.

Neuromotor Outcomes

An association between SGA and/or FGR with CP in children born preterm has been reported from CP registers, for example, from the Surveillance of Cerebral Palsy in Europe (SCPE), who found that those born with BW for GA below the 10th percentile (using fetal growth standards) had a higher risk for CP than those with a BW between 25th and 75th centile. This was found for the GA range of 32–41 weeks and was similar for all CP subtypes. The association between SGA at birth and CP, however, was not as clear for those born very or extremely preterm. Interestingly, a higher risk is also reported for those with BW above the 97th centile (11). Similar data are reported from other cohorts. For example, Jacobsson (49), using data from the CP register for West Sweden, after adjustment for maternal height, weight in early pregnancy, parity, ethnic origin and baby's sex, did not find an increased proportion of children who were SGA at birth in the preterm group (born <36+6 wGA), whereas in the term born group children with CP were significantly more likely to have been SGA at birth. It should be kept in mind that these are associations and that this does not necessarily mean that growth restriction causes CP. For very and extremely preterm children, the evidence suggests that other biological risk factors, in particular, focal preterm brain injury, pose a higher risk for severe neuromotor impairment than growth restriction. The findings of the studies reviewed here (**Table 2**) do not suggest that SGA and/or FGR pose a significant independent risk for CP in children born preterm, and this appears to be the case across the preterm gestational age range (7, 49, 58, 61, 66). Post-natal growth appears to be an important factor as, for example, indicated by the study by Franz et al. (58) in school aged children born <30 wGA and BW<1,500 g, who found that neuromotor outcome was associated with post-natal growth but not FGR, and this was still the case once analyses had been adjusted for important other biological risk factors including brain injury.

Neuromotor function in the absence of CP has been reported by several groups. The studies reviewed here report contradictory findings. Tanis (44), examined motor skills with the M-ABC in school aged children born 31–41 weeks of gestation in a population based cohort in the Netherlands, and did not find a difference in motor skills for the moderately preterm group between those who were AGA and those who were SGA at birth. Korzeniewski et al. (64), reported for the extremely preterm born ELGAN cohort no differences in motor function (assessed with the GMFC and MACS) at school age between those born after FGR and those without FGR. In another study in extremely preterm children Kan et al. (63), report M-ABC data from the Victorian Infant Collaborative study, a population based study of children born extremely preterm, and did not find an association between motor skills and FGR. However, they did see a significant association between head circumference at age 2 years and age 8 years with motor skills, suggesting that post-natal and post-neonatal brain growth is a crucial factor for motor outcome at school age, independently of presence of absence of FGR. In contrast to the findings by Kan (63), Tanis, and Korzeniewski (44, 63, 64) from 3 large population based cohorts, Raz et al. (67), report for a single center study of children born <34+6 wGA, using the PDMS-2, poorer gross and fine motor skills as well as performance IQ scores, for those with FGR compared to those born with appropriate for GA birth weight, and, importantly, an association between intrauterine growth and motor skills as well as Performance IQ even in those who had adequate standardized birth weight. Despite these inconsistent findings between studies, it appears to emerge that post-natal and post-neonatal brain growth is more important than intrauterine growth for neuromotor development in those

TABLE 2 | Included studies for preterm born children.

References	Study type	Definition of fetal growth	Participants	Neurology/neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Franz (58)	Single center study (Level 3 Neonatal Unit)	IUGR defined as BW < −2 SD according to British Growth Reference (59) expressed as SDS	Born < 30 wGA and BW < 1,500 g BY 1996–1999 *N* = 297 preterms admitted to neonatal unit Age at assessment: 5.4 y Those with neuromotor and sensory impairment included	Neurological examination: normal, mildly abnormal (minor neurologic signs such as broad gait, dysdiadochokinesis, or dysmetria), severely abnormal (any paresis with or without spasticity, cerebral nerve palsy, or ataxia) GMFCS for level of motor function *Findings:* Neuromotor outcome associated with post-natal growth, not IUGR	K-ABC (Mental Processing Composite, MPC) Analyses adjusted for GA, sex, multiple birth, ICH > Grade 3; PVL, ROP > Grade 3, ventilation > 7 days, language, maternal education	MPC associated with IUGR (BW), early neonatal weight gain, head growth post-discharge	Intrauterine growth (weight) and in-hospital weight gain predictor of cognitive outcome Post-natal weight gain but not IUGR associated with CP HC growth but not weight gain from discharge to follow-up predictor of cognitive outcome Combined contribution of IVH and prolonged mechanical ventilation greater than the combined contribution of growth
Guellec (7)	Population based; EPIPAGE study, 9 regions in France	*N* = 2,846 SGA = <10th centile (9.2%) Mildly SGA (MSGA) = 10th–<20th centile (9.6%) AGA =>20th centile Neonatal internal reference to approach *in utero* growth restriction, similar to Mamelle et al. (60) 2 preterm groups: <28 weeks GA (*n* = 828) 28–32 weeks GA (*n* = 2018) Term born (39/40 wGA) reference group (666 children) included at birth in the same regions	All live borns 24–32 weeks GA, born 1997 in 9 regions in France *n* = 2,846 Age at assessment: 5 y: 77% neurology; 65% cognitive assessment;71% behavior questionnaires 8 y: 61%	Categorized into presence of absence of cerebral palsy (SCPE criteria for CP used) *Findings:* **29-32 weeks GA group:** Cerebral Palsy: AGA 7.7%; MSGA 4.6%; SGA 3.2% **<28 weeks GA group:** Cerebral Palsy: AGA 14%; MSGA 11.1%; SGA 18.2%	**At age 5 years**: K-ABC: Mental processing composite score (IQ equivalent). Moderate cognitive deficiency: score between 70 and 84; severe cognitive deficiency: score <70 Behavioral problems: SDQ: inattention-hyperactivity, conduct, emotional, peer problems; total behavioral difficulties score **At age 8 years**: School questionnaire (school difficulties: special schooling institution or special school, special class in mainstream school, mainstream class) or low grades Analyses adjusted for sex, GA	**29–32 weeks GA group:** Cognitive deficit: AGA 28.7%; MSGA 41.8%; SGA 40.6% Behavioral problems: AGA 19.4%; MSGA 15.7%; SGA 23.5% School difficulties: AGA 18.4%; MSGA 32.18%; SGA 28 % **<28 weeks GA group:** Cognitive deficit: AGA 38.3%; MSGA 32.1%; SGA 37.5% Behavioral problems: AGA 23.7%; MSGA 27.3%; SGA 33.3% School difficulties: AGA 33.2%; MSGA 44.8%; SGA 35.3% Analyses controlled for socio-economic factors and sex	Growth restriction associated with adverse neurodevelopmental outcomes only in the 29- to 32-week GA group; both SGA and MSGA associated with an increase of cognitive deficiency, behavioral problems, and school difficulties. CP not associated with SGA

(Continued)

TABLE 2 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Guellec (61)	Population based; EPIPAGE study, 9 regions in France	4 categories: Symmetric growth restriction (SGR): HC and BW <20th centile and in the same percentile range; asymmetric growth restriction (AGR): at least 1 of HC and BW <20th centile and the other in a higher decile range. Two forms of AGR: head growth restriction (HGR), weight growth restriction (WGR). AGA: both BW and HC >20th centile	All live borns 26–32 wGA BY 1997 Age at assessment: 5 y (school performance at 8 y)	Categorized into presence of absence of cerebral palsy (SCPE criteria for CP used) *Findings:* No difference between groups for CP	K-ABC: Mental processing composite score (IQ equivalent), Moderate cognitive deficiency: score between 70 and 84; severe cognitive deficiency: score <70 Behavioral problems: SDQ: inattention-hyperactivity, conduct, emotional, peer problems; total behavioral difficulties score	SGR: higher risks of both moderate and severe cognitive deficiency. HGR: only higher risk for severe cognitive deficiency No difference between groups for behavior SGR: higher rate of school difficulties than AGA children Discussion	SGR associated with impaired cognitive and school performance Outcome of AGR differed according to HC: HGR associated with impaired cognitive function; WGR not associated with cognitive outcome No higher risk for CP in SGR
Kallankari (62)	Population based (regional cohort, Finland)	FGR defined as BW < −2 SD from mean of gestation-adjusted birth weight; documented FGR due to placental insufficiency by Doppler ultrasound and histological placental perfusion defect and lack of congenital infections or malformations	Born <32 wGA ($n = 154$); term born controls ($n = 90$) BY 1998–2002 Age at assessment: 9 y $n = 77$ preterms; $n = 18$ (23% FGR), $n = 27$ term controls Only preterms without CP and without cognitive impairment included	N/A	14 subtests from NEPSY II 6 subtests from WISC-III Mean scores for 5 domains calculated: visuospatial–sensorimotor processing, attention–executive functions, language, memory–learning and social perception Analyses controlled for GA, sex, maternal education	FGR only identified risk factor for impairment in language and memory learning skills in the preterm group; no difference between preterm AGA and FGR group for attention, executive functions, visuo-spatial, sensorimotor function	FGR independently predicts poor language, memory and learning skills
Kan (63)	Population based; Victorian Infant Collaborative Study	IUGR defined as BW < −2 SD according to British Growth Reference (59); expresses as Z-score	Born <28 wGA BY 1991–1992 $n = 179$ (2.2% IUGR); Age at assessment: 8 y Those with neurosensory impairment excluded	M-ABC 2 for assessment of motor skills (dexterity, ball skills, balance) *Findings:* HC or BW, or weight catch up in early childhood not associated with outcome, but, HC age 2 y and age 8 y associated with motor outcome	WISC-III for IQ WRAT-II for educational skills (reading, spelling, arithmetic) Analyses adjusted for potential biological and environmental risk factors (IVH grade 3 or 4, cystic PVL, surgery, post-natal steroids; maternal education, social class, other languages than English)	IUGR not associated with outcome at age 8 years HC or weight at birth, or weight catch up in early childhood not associated with outcome, but HC age 2 y and age 8 y associated with cognitive outcome	Intrauterine growth mostly unrelated to cognitive and other outcomes in preterm children without neurosensory impairment, but events between post-birth and age 2 years have an effect on brain growth and function, over and above effects of prematurity

(Continued)

TABLE 2 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Korzeniewski (64)	Multicenter, prospective, observational study (Extremely Low Gestational Age Newborns, ELGAN)	3 groups according to BW *z*-score (65) Severe FGR: <−2, Less severe FGR: ≥−2 and <−1 No FGR: ≥−1	Born <28 w GA *N* = 889 (93%) of original cohort BY 2002–2004 *n* = 52 severe FGR *n* = 113 less severe FGR Age at assessment: 10 y	GMFCS for gross motor function MACS for hand function *Findings:* FGR not associated with poorer motor function	DAS-II, NEPSY-II for general and specific cognitive functions (including executive function, memory, attention, verbal reasoning, visuo-motor precision etc.) WIAT-III for academic achievements (reading, spelling, numerical operations) OWLS for language (oral and written), Communication Function Classification Scale, and CCC-2 for communication skills SRS for social abilities, SCQ for screen for autism; ADOS-2 for autism diagnosis Analyses adjusted for sex and racial identity	Both severe and less severe FGR similar in having lower scores than no FGR peers in verbal reasoning, listening, comprehension, visuomotor precision, word reading, working memory, pseudoword decoding, and spelling subtests; more problems with social awareness and social cognition Severe FGR group lower scores than less severe FGR group for measures of auditory attention and response as well as inhibition switching, inhibition naming, as well as having higher scores on autism screen, and a variety of communication problems	Severe FGR poses increased risk of multiple cognitive and behavioral dysfunctions There is a positive relationship between severity of FGR and severity of cognitive impairment and behavior FGR not associated with poorer motor function
Morsing (66)	Single center study, Sweden	BW < −2 SD from mean of Swedish growth standards (53)	46 fetuses with IUGR and ARED umbilical artery blood flow; delivered <30 wGA at level III perinatal center BY 1998–2004 Follow-up: *n* = 34 preterm SGA with IUGR, ARED on umbilical arterial flow antenatally *n* = 34 preterm AGA (matched for sex, GA, BY) *n* = 34 term AGA Age at assessment: 5–8 y Those with CP not excluded	Information on diagnosis of CP and GMFCS level, hearing and visual function collected at time of cognitive assessment *Findings:* No difference in CP frequency	WPPSI-III; WISC-III (IQ) SDQ, Brown ADD Scale (total scores) for socio-emotional functioning and attention difficulties Analyses controlled for parental education, neonatal septicaemia, CLD, ICH>grade 3, PVL, post-natal steroids, ROP grade 3–5	FSIQ and VIQ lower in SGA/IUGR preterms than AGA preterms; PIQ not different; this was driven by poorer performance of male preterm SGA/IUGR children No difference in SDQ and ADD scores	Male preterm SGA children with IUGR and ARED blood flow *in utero* have poorer cognitive outcome than female preterms with IUGR and AERD No higher risk for CP
Raz (67)	Single center study	IUGR defined as BW < 10th centile; Z-score computed as the deviation of BW from the mean BW gestational age group, at delivery	Born 23–34+6 wGA (mean 28.6) BY 1991–2000 *n* = 25 IUGR *n* = 118 AG (appropriate growth) Age at assessment: 3–6 y Those with CP and ICH excluded	PDMS-2 for gross and fine motor skills *Findings:* Difference in gross and fine motor scores with IUGR having poorer motor skills	WPPSI-R for IQ scores PLS-3 for language skills (auditory comprehension and expressive communication) Analyses adjusted for sex, no significant difference in socio-economic class between groups	Full Scale IQ poorer in IUGR group; driven by difference in Performance IQ scores	Association between intrauterine growth and cognitive and motor outcome even within the population of preterm children who had adequate standardized birth weight

(Continued)

TABLE 2 | Continued

References	Study type	Definition of fetal growth	Participants	Neurology/neuromotor function	Cognitive/behavior Outcome measure	Cognitive/behavior findings	Conclusion
Tanis (44)	Population based regional cohort—subgroup (Longitudinal Preterm Outcome Project study), Netherlands	BW < −1 SD and birth weight > −1 SD according to GA; Dutch Kloosterman curve (45). Cut-off for SGA (> 1 SD, below the 16th percentile) according to Etude Epidemiologique sur les Petits Ages Gestationnels study	Born 31–41 wGA. AGA = 336; 216/336 moderately preterm SGA = 42; 216 32/42 moderately preterm BY 2002–2003 Age at assessment: 7 y	M-ABC 2 for assessment of motor skills (dexterity, ball skills, balance) *Findings:* No significant difference between groups	WISC III (Full Scale IQ) TEA-Ch: selective attention and Attention control RAVLT: Verbal memory NEPSY-II: Visuomotor integration BRIEF: Executive functioning in daily life Analyses adjusted for GA and sex	SGA children poorer attentional control, irrespective of GA No significant difference in other outcome measures	SGA children higher risk for difficulties with attentional control, irrespective of GA at birth. General cognitive, executive function, and motor skills not different between AGA and SGA

AGA, appropriate for gestational age; ARED, absent or reversed end-diastolic blood flow; AGR, asymmetric growth restriction; BW, birth weight; CLD, chronic lung disease; FGR, fetal growth restriction; GA, gestational age; HGR, head growth restriction; ICH, intracranial hemorrhage; IUGR, intrauterine growth restriction; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity; SGA, small for gestational age; SGR, symmetric growth restriction; w, week; WGR, weight growth restriction; y, year. ADOS, Autism Diagnostic Observation Schedule; CCL, Children's Communication Scale; CP, Cerebral Palsy; CSI, Child Symptom Inventory; Brown ADD, Brown Attention-Deficit Disorder Scales; BRIEF, Behavior Rating Inventory of Executive Function; CBCL, Child Behavior Checklist;; DAS, Differential Ability Scales; GMFCS, Gross Motor Function Classification Scale; K-ABC, Kaufmann Assessment Battery for Children; MACS, Manual Ability Classification Scale; M-ABC, Movement ABC for Children; MPC, Mental Processing Composite; NEPSY, A Developmental NEuroPSYchological Assessment; OWLS, Oral and Written Language Scales; PDMS, Peabody Developmental Motor Scales; PLS, Preschool Language Scale; RAVLT, Rey's Auditory Verbal Learning Test; SCQ, Social Communication Questionnaire; SDQ, Strengths and Difficulties Questionnaire; TeaCH, Test of Everyday Attention in Children; WIAT, Wechsler Individual Achievement Test; WISC, Wechsler Intelligence Scales for Children; WPPSI, Wechsler Preschool and Primary Scale of Intelligence; WRAT, Wide Range Achievement Test. The terms SGA, IUGR, FGR were used as in the study that is described.

who do not develop CP, which is also supported by Neubauer et al. (68), who examined 448 children born <32 w GA (19.9% SGA), and found that head size at age 3 months was the best predictor for motor (and cognitive) outcome at age 24 months. Visuo-motor skills at age 11 years were examined in the study by Kok et al. (69), for a cohort of preterm children (born <33 wGA) born FGR and who had antenatal Doppler studies performed; comparison was made between the group with and without "brain sparing." No significant differences were see in any of the tests (Beery-Buktencia Developmental test of Visuo-motor Integration, Motor Accuracy Test (MAT), Motor-Free Visual Perception Test (MVPT-R), and the balls skills subtest from the M-ABC).

Cognitive and Behavioral Outcomes

It is well-known that prematurity, in particular, very and extremely preterm birth, poses a risk for impairment of general cognitive abilities as well as difficulties in specific cognitive functions such as memory functions, processing speed, cognitive flexibility, attentional abilities, as well as socio-emotional behavior, all of which will affect for academic progress and peer relationships. Being born preterm and SGA and/or having FGR may be an independent risk factor for impairment of long term cognitive and behavioral outcomes, over and above other relevant biological and social risk factors. Long term outcomes of preterm birth are affected by a number of factors and it is often complicated to establish an independent effect of SGA and/or FGR on long term cognitive outcomes in the context of prematurity. Often, those who are SGA and/or have had FGR, also are those who, compared to those born AGA, have more other neonatal morbidities that have been show to affect outcome (such as chronic lung disease, neonatal sepsis, etc.), which has been illustrated, for example, by the large EPIPAGE study (61). In addition, often, there are no detailed fetal growth measures or information on fetal Doppler studies available, which can make it difficult to establish the exact cause of a preterm infant's SGA birth weight. Direct comparison of study findings is difficult since slightly different criteria for definition of SGA and/or FGR are used in different studies. In addition, although most studies have assessed general cognitive abilities with either the Wechsler Intelligence Scales (44, 62, 63, 66, 67), the Kaufmann ABC (7, 58, 61), or the NEPSY assessment (62, 64), different studies focused on different specific cognitive functions, and there is a large variety of different tests used. Furthermore, in some studies participants with neurosensory impairment were not excluded from analyses on cognitive and behavioral function, which complicates comparison with those studies in which only those without neurosensory impairment were assessed. However, overall, the studies reviewed here suggest that preterm children born SGA and/or having had FGR, compared to their AGA preterm peers, are at increased risk of developing cognitive and behavioral difficulties, although some study findings are inconsistent. With the exception of the study on the Victorian Infant Collaborative Study cohort (63), who did not find an association between FGR and cognitive abilities at school age, but between post-neonatal head growth and cognitive development, the large multicenter or population based studies reviewed here

(7, 44, 61, 64) report an effect of FGR (it has to be kept in mind that in most studies SGA was used as a surrogate marker for FGR) on long term cognitive function, and, in the majority of the studies, this was still the case once other biological and social risk factors are considered. Guellec (7), reported school age outcome data for the EPIPAGE cohort (GA range 24–32 weeks) and divided the SGA participants into 2 groups, mildly SGA and SGA, and also formed 2 GA groups (<28 wGA, 29–32 wGA). They found impaired cognitive function, behavioral problems, and school difficulties at school age were more frequent in both the mildly SGA and the SGA born children in the 29–32 wGA group but not in the more immature GA group.

Korzeniewski et al. (64), examined school age outcomes for the extremely preterm (<28 wGA) born ELGAN cohort, focusing on general and specific cognitive abilities (including executive function, memory, attention, verbal reasoning, visuo-motor precision), communication abilities, and socio-emotional behavior. They did find overall a positive relationship between severity of FGR and severity of cognitive impairment and behavior for most areas assessed, and, in addition, that those with the most severe FGR also had more problems than those with less severe FGR in auditory attention, inhibition switching, inhibition naming, higher scores on autism screen, and a variety of communication problems. Kallankari et al. (62), followed a Finnish cohort of children born at a more mature GA (<32 wGA) for whom FGR due to placental insufficiency was documented by Doppler ultrasound and histological placental perfusion defect. They reported that FGR independently predicted poor language, memory and learning skills, but did not find an effect of FGR on attention, executive function, visuo-spatial, or sensorimotor function. For a cohort with a larger range of GA at birth (31–41 wGA), Tanis et al. (44), established that those born SGA were at higher risk for difficulties with attentional control, irrespective of GA at birth, but that there was no difference to those born AGA for measures of general cognitive abilities, executive function, visuomotor integration, or memory function. These findings on attention difficulties in children born SGA and/or FGR are consistent with those from a large nationwide case-control study in Finland that investigated associations between prematurity and fetal growth in relation to Attention Deficit Hyperactivity Disorder (ADHD) (70). This study found that prematurity was a risk factor for ADHD across the preterm gestational age range, and that with lowest weight for GA (< −2 SD) had the highest risk for ADHD, together with those being more than 2 SD large for GA.

Some studies have investigated childhood outcomes of preterm children born SGA and/or FGR for whom information on umbilical artery blood flow was available. Morsing et al. (66), examined outcome in a cohort of fetuses with FGR and absent or reversed end-diastolic umbilical artery (AERD) blood flow who were delivered before 30 wGA, and compared them to a control group of preterm infants of the same GA born with AGA birth weight. At school age those with AERD had poorer full scale and verbal IQ than those born AGA and this difference was driven by the poor performance of male participants in the FGR/AERD group. These findings are inconsistent with those of Valcamonico et al. (71), who found a higher incidence of severe neurological problems (CP, visual and/or hearing impairment) but no difference in general cognitive abilities in a group of very preterm born (mean GA at birth 33.3 weeks; 35 % SGA with BW <5th centile) school aged children who had AERD when compared to a group of children (mean GA at birth 30.8 weeks; 38% SGA) who did not have AERD. It is possible that the difference in outcomes between these studies are explained by the different proportions of children having been SGA at birth, despite all having had AERD; furthermore there are differences between studies in the methodology of the Doppler studies and definition of SGA and/or fetal growth. van den Broek et al. (72), compared behavior in a large cohort of 11 years old children born preterm (<33 wGA) in whom weekly antenatal Doppler studies were done to assess the ratio between umbilical and cerebral pulsatility index (U/C ration) for assessment of "brain sparing." In this study, rather than using centile curves, fetal growth ratio was defined as the ratio of the observed birth weight to the expected mean birth weight for gestational age, based on Dutch intrauterine growth curves. At age 5 years significant difference in the proportion of those with Full Scale IQ <85 in the group with "brain sparing," but no significant difference for mean FS IQ between the groups. At age 11 years no significant association was seen between parent and teacher rating of behavior on the CBCL and U/C ratio after controlling for neonatal variables and IQ at age 5 years, indicating that "brain sparing" was not an independent risk factor for behavioral difficulties at age 11 y.

Most of the existing studies in term born babies use growth or birth weight <10th centile, often based on different growth standards, as definition of FGR. Using more stringent FGR bands may be useful in future work in order to examine in more detail whether there is an effect of only severe FGR on development. Kan et al. (63) studied long term cognitive outcome in extremely preterm born children, using stricter criteria for FGR (< −2 SD) and did not find an association between FGR and outcome, but strong associations between post-natal head growth and outcome, which emphasizes the importance of head growth, very likely independently of the severity of FGR. The importance of post-neonatal head growth for cognitive development in children born SGA and/or FGR has also been highlighted by the study by Franz et al. (58), who reported for a single center cohort born <30 wGA that FGR, in-hospital weight gain, and post-neonatal head growth (but not weight gain) predicted general cognitive abilities at school age (63).

CONCLUSIONS

Overall, existing studies are heterogeneous, for example, with regards to definitions of growth restriction and small for gestational age, time of onset of FGR, inclusion and exclusion criteria, outcome measures, all of which makes comparison between studies difficult.

However, in summary, the literature reviewed here suggests that cognitive development in term born children is not hugely

impacted by them being born SGA or FGR, particularly in the case of general intellectual function, on which the majority of studies have been conducted. There is some limited evidence that term born growth restricted children may experience problems with attention, and such difficulties may have more impact on children's learning and behavior as they progress through school, suggesting that this would be a useful area for further work. The risk for developing CP is higher in children born SGA with FGR; currently there is contradictory information available on neuromotor function in the absence of CP.

In contrast, preterm born SGA and/or FGR children are at great risk of poorer general intellectual functioning than their AGA counterparts. These children are also at risk for difficulties with attentional control, and some studies suggest that memory, executive function, and communication skills are also affected. It does not appear that SGA and/or FGR *per se* pose a higher risk for CP; information on motor outcome in the absence of CP is, similar to the term born children, somewhat contradictory.

Post-neonatal head growth and head growth in infancy and early childhood emerges as an important factor for long term neurodevelopmental outcome.

There is some inconsistency between the conclusions of our review of school aged children and those studies that report follow up data acquired in the toddler years. This could result from certain cognitive difficulties taking time to emerge and/or these being more subtle problems that are nevertheless clinically and functionally important. It is important to continue to follow up children into the school age years because these difficulties may take time to emerge, and may be more visible in the more demanding school environment.

AUTHOR CONTRIBUTIONS

BV and CE have made equal contributions to this work. Both provide approval for publication of the work and agree to be accountable for all aspects of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 29 March 2019
doi: 10.3389/fendo.2019.00188

Knowledge Gaps and Emerging Research Areas in Intrauterine Growth Restriction-Associated Brain Injury

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *26 October 2018*
Accepted: *06 March 2019*
Published: *29 March 2019*

Citation:
Fleiss B, Wong F, Brownfoot F, Shearer IK, Baud O, Walker DW, Gressens P and Tolcos M (2019) Knowledge Gaps and Emerging Research Areas in Intrauterine Growth Restriction-Associated Brain Injury. Front. Endocrinol. 10:188. doi: 10.3389/fendo.2019.00188

Intrauterine growth restriction (IUGR) is a complex global healthcare issue. Concerted research and clinical efforts have improved our knowledge of the neurodevelopmental sequelae of IUGR which has raised the profile of this complex problem. Nevertheless, there is still a lack of therapies to prevent the substantial rates of fetal demise or the constellation of permanent neurological deficits that arise from IUGR. The purpose of this article is to highlight the clinical and translational gaps in our knowledge that hamper our collective efforts to improve the neurological sequelae of IUGR. Also, we draw attention to cutting-edge tools and techniques that can provide novel insights into this disorder, and technologies that offer the potential for better drug design and delivery. We cover topics including: how we can improve our use of crib-side monitoring options, what we still need to know about inflammation in IUGR, the necessity for more human post-mortem studies, lessons from improved integrated histology-imaging analyses regarding the cell-specific nature of magnetic resonance imaging (MRI) signals, options to improve risk stratification with genomic analysis, and treatments mediated by nanoparticle delivery which are designed to modify specific cell functions.

Keywords: growth restriction, neurobiology and brain physiology, brain development, neuroprotection, neuroinflammation

INTRODUCTION

Intrauterine (or fetal) growth restriction (IUGR, FGR) is caused by a heterogeneous set of maternal and fetal clinical pathologies. Stillbirth, neonatal mortality and poor neurological, and cardiovascular outcomes are all too common consequences of IUGR. We do not have the clinical tests to reliably predict the onset of IUGR, or even to reliably detect it, when present in late gestation. A diagnosis of IUGR is simply the indication that the infant has a birth weight below their genetically predetermined potential, with no etiological meaning. Our understanding of the

clinical progression of IUGR including the poor neurodevelopmental outcomes has increased due to improved imaging techniques, such as Doppler velocimetry and magnetic resonance imaging (MRI). Nevertheless, as a research community, we are still striving to understand the pathological mechanisms leading to the various subtypes of IUGR (discussed below). Increase of this knowledge is crucial for tailoring therapies to prevent or treat IUGR and, in particular to reduce brain injury. Highlighting the need for a continuing collective research efforts is that, depending on the specific definition of IUGR applied (discussed below), the incidence of IUGR is between 3 and 9% of pregnancies in high resource settings, but horrifically, in low-resource settings, the rates are as high as 30% of pregnancies (1).

The purpose of our review, written in a narrative form [as defined in McGaghie (2)], is to highlight the gaps in our clinical and translational knowledge, and the weaknesses of our research methods that hamper our collective efforts to improve the neurological outcomes of those infants diagnosed with IUGR. Our opinions and interests are diverse—as authors we include clinicians working as obstetricians, neonatologists, and neonatal neurologists, plus researchers with expertise in fetal physiology and developmental neurobiology. We will focus on new cutting-edge tools and techniques that might provide novel insights into this disorder of fetal growth, and on technologies that offer the potential for better drug design and delivery. We will cover topics including maximizing the benefit of data from scarce human post-mortem tissues, lessons from advanced histological analyses regarding the cell-specific nature of MRI signals, options to improve risk stratification with genomic analysis, and new treatments based on delivery of nanoparticles designed to modify cell-specific functions.

WHAT IS IUGR?

The issue of an optimal definition for IUGR has been called "one of the most common, controversial, and complex problems in obstetrics" (3). Simply, IUGR is usually recognized when an infant appears to have failed to grow to its expected size—based on it genetically pre-determined potential. Most surprisingly though, there is no universally applied definition of what threshold of body weight clearly defines an infant as IUGR, with birth weights at or below the 10th or the 3rd centile, and concomitant changes in placental blood flow and gestational age all taken into account with varying frequencies across health care centers even within countries. An advance in bringing a universally recognized definition to the field was made with the recent publication of a Delphi procedure resulting in an agreed definition of IUGR (4). However, although a Delphi procedure is a well-established method for finding consensus, only 45 participants from across the entire world took part; 54% of these being from Europe, encompassing 37 member states with various population risk factors and health care paradigms. In addition, Asia and Australia were grouped as a single entity with only 10 opinions received from these diverse areas of the world. The Delphi procedure presented clinical variables and outcomes already known to be associated with IUGR and asked the participants to rank or stratify the importance of these. The end result was that somatic growth indices, umbilical artery pulsatility index, and absent end-diastolic flow were the favored diagnostic criteria. At this point we would like to highlight there is no pathophysiological meaning within the term IUGR (or FGR) as IUGR ultimately is a symptom, like the diagnosis of microcephaly (i.e., brain growth at or below two standard deviations from the mean). The term IUGR (or FGR) is as useful for understanding the disorder as if we used the term PRBS (poorly regulated blood sugar) in place of diabetes. The Delphi procedure offers no clarification on whether it is possible to stratify patients based on the underlying cause of their IUGR, although admittedly, this was not its purpose. However, we need to revaluate the criteria currently used to determine IUGR, (e.g., somatic growth, umbilical blood flow) some of which might well-reflect compensation and adaptation to an underlying, unidentified causal mechanism. Additional measurements could include blood-based biochemical measures of inflammation, placentally-derived biomarkers, postnatal blood pressure and urine parameters, and additional biometrics such as skin folds, and skeletal phenotype. Meta-analysis of clinical data banks, together with studies on stored chorionic villi and maternal blood retained after routine clinical testing, and (albeit, complicated) the recruitment of further large prospective cohorts—could provide us with biochemical parameters that closely reflect the underlying causes of reduced fetal growth, and how these stratify with risk across the lifespan.

How we name a disease has a significant bearing on how we then think about it, and ultimately, how we model and aim to treat it, so these issues of universal nomenclature are important; see commentaries by Dammann et al. (5), and McIntyre et al. (6) for the importance of classifying known causes, and not symptomatic outcomes. A recent summary of the definition(s) of IUGR as they have evolved over the past 30 years highlights three facts: (1) that a significant number of clinical studies on IUGR (11%) gave no definition of IUGR; (2) that ultrasound measurements of fetal biometrics, but not Doppler measurements of placental and/or fetal brain blood flows, have been increasingly used to define IUGR, and; (3) that overall the primary, consistently used characteristic of IUGR diagnosis is birth weight (7). This last observation highlights that we are missing opportunities to identify the early prenatal events that lead to IUGR, and therefore of developing our approaches to increase the efficacy of interventions, and the opportunity to prevent, rather than repair brain damage and therefore to improve neurological outcomes for these infants via early identification. A further confounder in diagnosing IUGR is the lack of accurate gestational dating, as in many countries (including the USA) routine ultrasound dating scans are not available and estimates based on last menstrual period are generally unreliable.

Maternal and fetal genetic factors are responsible for >50% of the variance in birth weight (8). As such, is it critical to separate healthy infants that are small for gestational age (SGA) due to inherent genetic factors from those with reduced growth that are at risk of sustaining neurological injury and metabolic

dysfunction. Personalized growth charts, based on maternal and fetal characteristics (such as age, body weight, fetal sex, and ethnicity), have improved our ability to differentiate IUGR and SGA infants [for a commentary see (9)]. While correcting for maternal parameters seems appropriate, perhaps correcting for ethnicity might normalize a socially disadvantaged group with higher rates of growth restriction and poor obstetric outcome. This was perhaps best reflected in INTERGORWTH 21 which recruited over 20,000 pregnant women from numerous countries around the world in regions where the health and nutrition of the mother were met and antenatal care was adequate, and they demonstrated that the growth parameters were similar regardless of ethnicity.

IUGR is also classified as symmetric or asymmetric IUGR (**Figure 2**), dependent on the ratio of the head circumference to the abdominal circumference, which is increased in asymmetric IUGR. This categorization is based on the idea of brain sparing arising (typically) from late-onset IUGR. Early-onset IUGR (before 32 weeks post-conceptional age [PCA]) is more often associated with the symmetrical form of IUGR, and late-onset IUGR (after 31 + 6 weeks PCA) is more often associated with asymmetric IUGR. For further details on brain sparing and a discussion on the consequences and relationship between adaptive to maladaptive (compensatory vs. decompensatory) processes, see the section on "Causes of IUGR," below. There is also a third phenotype of IUGR reflecting the accumulated effects of early *and* late IUGR risk factors (10). This mixed phenotype is observed predominantly in pregnancies complicated by peri-conceptual malnutrition, and then by placental dysfunction later in pregnancy. These infants are suggested to present with symmetric growth, but with severe signs of malnutrition, such as high numbers of scapula skin folds (10).

WHAT ARE THE OUTCOMES ASSOCIATED WITH IUGR?

IUGR fetuses are at increased risk of stillbirth, fetal compromise, early neonatal death, and neonatal morbidity (11). A vast literature including many works from Winder et al. has demonstrated that the availability of physiological resources that support growth *in utero,* which include not only maternal nutritional status (12) but also placental size, shape, and metabolic efficiency, have effects that continue to have an impact on health throughout childhood and adult life (13, 14). Indeed, IUGR-born infants are prone to a range of health problems, including increased risk of cardiovascular diseases and neurodevelopmental disorders (15, 16).

It has been difficult to compile outcome data from the many studies on IUGR, because in addition to the fact that IUGR includes an inconsistent, heterogeneous set of clinical characteristics and underlying etiologies, postnatal data reporting includes further inconsistencies in patient selection and outcome parameters. To enable future trials to measure similar meaningful outcomes, the Core Outcome Set for GROwth restriction: deVeloping Endpoints (COSGROVE) consortium is developing two core outcome sets—one for prevention and the other for treatment of IUGR (17). These guidelines will ensure the collection and reporting of a minimum dataset, agreed by stakeholder consensus that will reduce inconsistencies in the reporting of outcomes across relevant trials. For comprehensive reviews on IUGR-related brain injury, including specifics related to the type and severity of IUGR and outcomes, we refer the reader to Miller et al. (18), Tolcos et al. (19), and Gilchrist et al. (20). Over all, infants that were born IUGR have a significantly increased risk of motor and sensory neurodevelopmental deficits, cognitive and learning impairments, and cerebral palsy (see papers above). In particular, in near-term and term infants the rates of cerebral palsy are higher in IUGR infants (16.5%) than the rates in infants exposed to birth asphyxia (8.5%) or inflammation (4.8%) (21). A common comorbidity of IUGR is preterm birth, and this confounds our understanding of the specific effects of IUGR on brain development and function (discussed below). In addition, the risk of delivering an IUGR baby is higher for woman with chronic hypertension, pre-eclampsia, low socioeconomic status, overt diabetes, anemia, gestational diabetes mellitus, low pre-pregnancy body mass index, or hypothyroidism (22). Whether each of these risk factors leads to a specific phenotype of outcome for the IUGR infant is still a matter that requires considerable study. A number of these risk factors and their causal role in IUGR is discussed further below.

WHAT ARE THE CAUSES OF IUGR?

The primary cause of IUGR is widely considered to be placental insufficiency; i.e., inability of the placenta to adequately support fetal growth. However, the causes of placental insufficiency are many and over-lapping, and include constricted spiral arteries and increased coagulation leading to fetal hypoxia (as in maternal hypertension), and inappropriate substrate availability due to maternal under-nutrition or over-nutrition (22, 23) (see **Figure 1**). For the purposes of this review we won't discuss in detail the relationship between IUGR and maternal drug use (alcohol, tobacco, cocaine etc) or the association with infectious agents, the so-called "TORCH" infections; Toxoplasmosis, Other (syphilis, hepatitis B, varicella-zoster virus, human immunodeficiency virus [HIV], parvovirus B19, enteroviruses, lymphocytic choriomeningitic virus etc.), Rubella, Cytomegalovirus, and Herpes simplex virus. However, these infections and exposure to environmental toxins often lead to a complex constellation of outcomes (microcephaly, facial abnormalities, intracranial calcifications, rash, jaundice, hepatosplenomegaly, elevated transaminase concentrations, and thrombocytopenia) which, although low in incidence in high-income settings (24), are significant in low resource settings (25).

Late vs. Early Onset IUGR and the "*Head-Sparing*" Effect

In addition to linking IUGR to specific pathological processes, as discussed above we also classify IUGR as symmetric or asymmetric (**Figure 2**), dependent on the ratio of the head circumference to the abdominal circumference of the infant. Asymmetric IUGR is the result of "brain sparing," a process

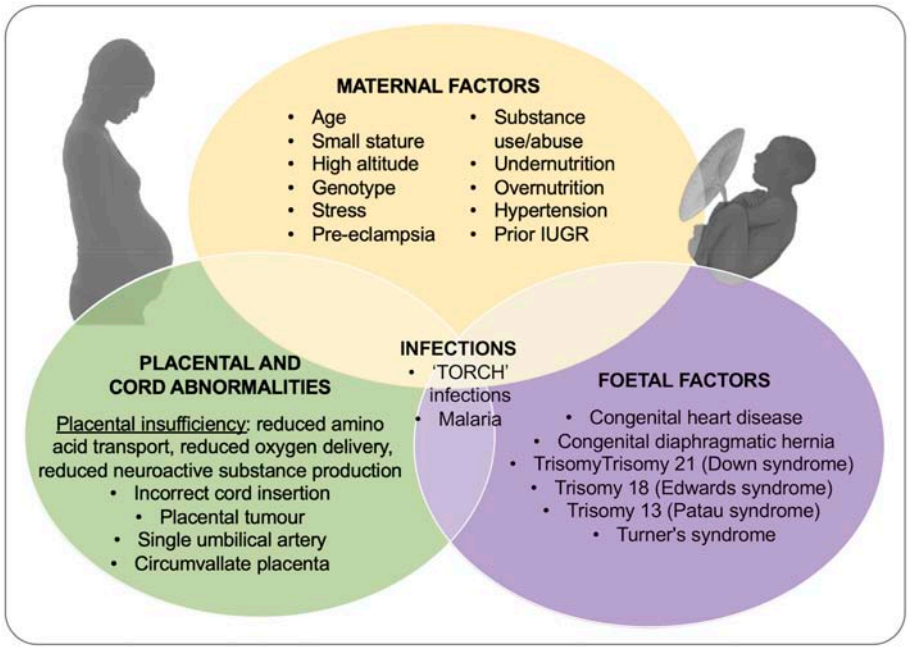

FIGURE 1 | Outline of the causes of IUGR including contributions from maternal, placental and umbilical cord, and fetal dysfunction or injury. Adapted from Vijayaselvi and Cherian (22) and Gaccioli and Lager (23).

whereby brain growth is less affected than body growth due to the redistribution of cardiac output. While brain sparing does not completely prevent the damaging effects of IUGR on brain development (26, 27), it is nonetheless associated with better neurological outcomes than when brain sparing does not occur (28). It is also worth noting that mortality is higher in IUGR with symmetric growth even after adjusting for possible cofounding factors (26). Brain sparing can be detected prenatally based on the Doppler pulsatility index (PI) in the middle cerebral artery (MCA); PI is reduced by the decreased cerebral resistance which allows a greater fraction of the cardiac output to perfuse the brain. However, this compensatory process of blood distribution can become "decompensatory" because the increase of brain blood flow and blood volume themselves become damaging (29). Understanding when and how the alteration of relative cerebral blood flow is switched from a compensatory to decompensatory response is clearly important for devising the most appropriate interventions and therapies. It is worth noting that brain sparing does not reduce the consequences of IUGR on later health, such as increased adiposity, diabetes, and cardiovascular risks, etc [reviewed in Sehgal et al. (30) and Devaskar and Chu (31)].

Maternal risk factors, especially diabetes, high body-mass-index and hypertension, are relevant risk factors for IUGR across both high- and low-income settings. This blurring of income-related demarcations is driven in part by changes in diet and lifestyle, such as adoption of a "western-style" diet and changes in the nature of "work" with more people employed in sedentary activities in urban centers across continents. An illustration of this is the increased risk for an IUGR infant when a woman is diabetic; in Africa the rate of diabetes in adults 25–64 years of age is at 15%, in India 9%, and in Australia 5% (WHO, heath-topics, 2018). An increased body-mass-index is also a major risk factor for gestational diabetes that also associates with poor fetal outcomes. The effects of diabetes on the placenta occur irrespective of the cause of diabetes and the careful management of blood sugar, and include thickening of the trophoblast basement membrane (32), which impairs oxygen and nutrient delivery. Diabetes is associated with a higher release of placental cytokines such as leptin, tumor necrosis factor-α (TNF-α), and Interleukin-6 (IL-6) [see Pantham et al. (33)]. Over-nutrition in the (apparent) absence of co-morbidities is also associated with poor placental development/function and poor fetal outcomes [recently reviewed in Howell and Powell (34)], and this has also been demonstrated in experimental models, including sheep, mouse, rat, and rabbit (35–37).

An important link between maternal overnutrition, poor placental development, and poor brain development is inflammation. Obesity is a known driver of systemic inflammation and neuroinflammation (see microglial section, below) (38), and a precise regulation of the maternal and fetal immune system is required for proper placental function and fetal brain development. Of note, adipose tissue produces adipokines, including the pro-inflammatory factors TNF-*a*, IL-6, and MCP1 (macrophage chemotactic protein 1 also known as CCL2). This is considered to explain (at least, in part) why maternal obesity is associated with higher levels of circulating inflammatory mediators during pregnancy and dysregulated placental nutrient transport (39, 40); such systemic and placental inflammatory effects can be reproduced by over-nutrition in pregnant sheep, leading to IUGR (41, 42).

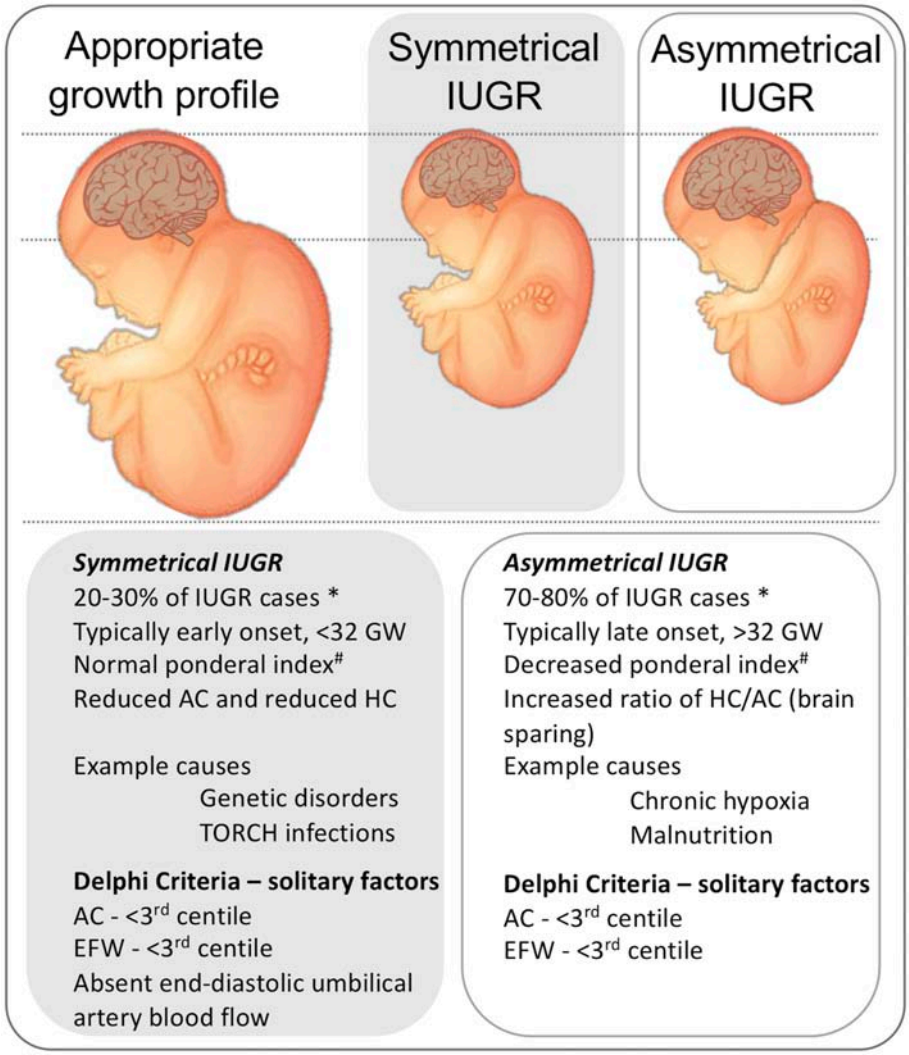

FIGURE 2 | Representation of the physical presentation of symmetrical and asymmetrical IUGR and a short list of clinical characteristics and causes. * note that incidence data are from high-resource settings. A third phenotype is proposed in low-resource settings, that includes characteristics of malnutrition and late gestation placental insufficiency (10), not shown. # Ponderal index, (birth weight (g)/length(cm)^3 × 100). HC, head circumference. AC, Abdominal circumference. GW, gestational weeks. AC, abdominal circumference. EFW, Estimated fetal weight. Delphi criteria from Gordijn et al. (4).

Preeclampsia increases the risks for IUGR 4-fold and is a leading cause of maternal and fetal death. Preeclampsia is a condition of vascular endothelial dysfunction and vasospasm that occurs after 20 weeks of gestation that has its origins in inadequate trophoblastic invasion of the uterine vascular bed, in particular, of the spiral arteries. World-wide, preeclampsia occurs in 8–10% of pregnancies and this rate is consistent across high, middle-, and low-income settings. The factors associated with the onset of preeclampsia include pregnancy at a young or advanced age, high maternal body-mass-index and psychosocial stress. Preeclampsia is usually associated with IUGR, but early onset preeclampsia may be associated with an enlarged placenta and over-large birth weight. While reports show there are differences in placental function and biomarkers between IUGR and preeclampsia, there is overlap between the outcome in these disorders and understanding the similarities and differences will be useful for managing maternal and fetal risk (43–47).

In low resource settings malnutrition is a leading causal factor in IUGR, and all too often these IUGR infants are also delivered preterm adding to the burden of mortality and neurodevelopmental injury (48, 49). Malnutrition is linked not only to economic factors, but also to cultural and social norms that include young maternal age, and repeated and closely spaced pregnancies. UNICEF estimates that in the most developed countries 7% of babies are of low birth weight (encompassing prematurity and IUGR predominantly), whereas in less developed, and the least developed countries, rates of low birth weight deliveries increase to 16.5 and 18.6%, respectively, representing more than 22 million babies annually. Although great improvements in health and well-being are being made,

especially as part of the Millennium Development Goals, specific focus on how to overcome poor neurological outcomes for the infants born to these mothers is still greatly needed. For more details on IUGR in less well-developed countries please refer to these references (1, 10, 48–52). Trials of nutritional supplements (micronutrient-supplemented protein, balanced calorie supplements etc.) in women with an IUGR pregnancy identified in the 2nd trimester have failed to improve outcomes, and overall the literature supports pre- and peri-conceptional nutrition as a major determinant of fetal development and pregnancy outcome [reviewed in Liberato et al. (53)].

WHAT ARE THE PRIMARY NEUROPATHOLOGICAL PROCESSES IN IUGR?

Placental Dysfunction

Early-onset IUGR is associated with high impedance uteroplacental perfusion and elevated umbilical artery blood flow resistance as measured by Doppler ultrasound [reviewed in detail by Dall'Asta et al. (54)]. Late-onset IUGR is more common, occurring in 70–80% of IUGR cases and the diagnosis and monitoring of late-onset IUGR has been recently reviewed by Figueras et al. (55). Overt placental pathology may be mild, or even absent in late-onset IUGR, and the Doppler parameters of umbilical artery blood flow may even be normal, but changes in brain blood flow dynamics, such as fetal middle cerebral artery impedance, may be reported indicating compensatory changes to cardiac output indicative of established IUGR. In addition to changes in uterine artery blood flow indicating high vascular resistance, placental studies reveal the important contribution of placental infarcts, and changes in placental amino acid and micro-nutrient transporters, and pathways for growth factor production, further support the concept of "functional" placental insufficiency of IUGR (56, 57). Based on these human studies, animal models of IUGR have been created that recapitulate aspects of the human condition (discussed below), and by and large demonstrate the link between the deprivation of oxygen, glucose, amino acid, and growth factors to the slowing of fetal growth. In addition to this it is now apparent that the abnormal change in placental metabolism results in increased production of reactive oxygen species (ROS), aberrant activation of the complement cascade, re-programming of microglial phenotype (see below), and changes in the trajectory of brain maturation that include dysregulated neural cell proliferation, slower maturation of oligodendrocytes resulting in hypomyelination, and increased programmed (apoptotic) cell death (58–61). We refer the reader to publications describing the mechanisms underpinning the neuropathology of IUGR for more details on these processes (19, 62, 63).

In addition to the somewhat obvious link between the placenta as a source of cytotoxicity, there is the possibility that growth promoting cytokines and neurotrophic growth factors are altered with placental insufficiency. In addition to somatic growth factors such as IGF1, the placenta is a source of pregnene and androgen steroids that directly, or via metabolites such as allopregnanolone, promote, and protect fetal brain development by promoting fetal sleep and quiescence [(64);and references within (65)]. It has been hypothesized the allopregnanolone is an endogenous neuroprotectant, via its effect of global inhibition of CNS activity, that protects against antenatal brain injury (66–68).This field of steroid research has given rise to the notion that there is a "placenta-brain axis" that reflects the co-ordinate development of the fetal adrenal gland, liver, and steroid metabolizing functions of the placenta (69, 70). Neurosteroids derived from progesterone such as allopregnanolone interact with GABA-A receptors and increase central nervous system (CNS) inhibition (71, 72). In uncomplicated pregnancies there are high levels of neurosteroids such as allopregnanolone in the fetal brain immediately before birth, but these levels fall rapidly with removal of the placenta because they are cleared quickly from the circulation, and have a half-life of only minutes (73). In the placenta of infants born preterm the activity of these neurosteroidogenic pathways is reduced, raising the possibility that the prenatal and postnatal paucity of allopregnanolone might affect brain development in these infants (74). In the serum of pregnant women carrying an IUGR fetus, levels of allopregnanolone are lower (75), and in animal models of IUGR the neurosteroid systems (genes and proteins) are lower in the fetal brain (76, 77). Direct inhibition of allopregnanolone production during development causes brain injury (78, 79) and lasting behavioral deficits (80). It is thus reasonable to suggest that progesterone, and even allopregnanolone, should be replaced in preterm and IUGR infants to improve neurological outcomes (81). For example, Ganaxolone is a synthetic analog of allopregnanolone that has a long half-life, has shown promise as a seizure therapy in adults and children refractory to anti-seizure treatments, and in animal studies does not alter fetal viability, neonatal growth, and is without teratogenic or genotoxic effects [reviewed in Hirst et al. (81)]. Ganaxolone therapy should (like all drugs) be trialed in large animal models (powered for sex and with long term outcome) of the various types of IUGR that can now be modeled—for example, by delayed onset chronic hypoxia such as the single artery ligation (SUAL) sheep model (82); early onset placental insufficiency in piglets—(60); maternal overnutrition before and during pregnancy in the sheep—(83). An important layer of safety data that needs to be obtained is the specific interaction that early treatment with ganaxolone might have on the developmental switch of GABA receptors from excitatory to inhibitory (84). This event is predicted to be before 26 weeks' gestation in humans and it might be expected that before this time that ganaxolone would induce, not supress, neuronal activity.

Of the amino acids transported and metabolized by the placenta, tryptophan has a special significance (85). It is an essential amino acid, but protein synthesis accounts for only a minor part of its fate, the greater part being committed to the kynurenine and serotonin pathways. Conversion of tryptophan to kynurenine via indoleamine 2,3-dioxygenase (IDO) in early pregnancy may be important for immune suppression and acceptance of the conceptus as an allograft (86). Later in pregnancy the synthesis of kynurenine may be more important because it is the precursor of kynurenic acid, a physiological

glutamate receptor antagonist, which acts as a neuroprotectant (87). Both gene and protein expression of IDO and tryptophan 2,3-dioxygenase (the second enzyme in the kynurenine to tryptophan conversion) are significantly lower in IUGR-affected placentas compared with controls [reviewed by, (88)]. IDO is an oxygenase, and its activity is downregulated in reduced oxygen conditions; demonstrated in *ex vivo* first and third trimester human placental explants exposed to lower oxygen (5–8% O_2) or higher oxygen (20% O_2) conditions. Exposure to lower oxygen levels reduced IDO mRNA and protein expression, and other kynurenine pathway enzymes and kynurenine output was also significantly reduced (88). Inflammatory mediators, such as TNF-α, IL-1β, and interferon-gamma, induce IDO expression thereby increasing tryptophan degradation to kynurenine, but with the result that pro-oxidant (e.g., 3OH-anthranilic acid) and glutamate agonist metabolites (e.g., quinolinic acid) are produced (85). The impact of IUGR on the placental metabolism of tryptophan is not fully understood, but it can be seen from the above that placental insufficiency could have a significant impact on the fetal brain via alterations in the placental degradation of tryptophan.

Tryptophan is also the precursor for the synthesis of serotonin (5-HT). Abnormal levels of brain 5HT have been linked to neurodevelopmental disorders such as autism spectrum disorder (ASD) (89), but when these abnormalities arise in humans (i.e., antenatally or postnatally?) is unclear. There is evidence from mouse pregnancies that placental 5-HT has an important role in early fetal brain development, in that the 5-HT needed for early forebrain development initially comes from the placenta (90). In early neurodevelopment 5-HT functions to regulate a number of key processes, including cell proliferation and neuronal differentiation, migration, and synaptogenesis (89), and experiments in the mouse clearly show the free entry of 5-HT into the immature brain. However, by late gestation, there is a decrease of placental 5-HT synthesis in humans and mice as the raphe nuclei in the midbrain become competent and 5HT axons reach the forebrain (90). This co-ordinate change of 5-HT synthesis between the placenta and brain really does suggest the presence of a "placenta-brain axis" which should be investigated more fully in experimental settings using where IUGR, placental insufficiency, and preterm birth can be modeled in animals with more relevance to human pregnancy. Specifically, an issue with traditional mouse studies is that the fetus is delivered at a stage of brain development equivalent to the start of the second trimester in the human; this makes studies of the last trimester fetal-placental axis impossible. However, peripherally synthesized 5-HT does not freely cross the blood-brain barrier in more fully developed (i.e., adult) brains (91). Goeden et al. (92) have also demonstrated the effects of mild maternal inflammation on placental tryptophan catabolism to 5-HT. Their findings suggest that maternal inflammation during human pregnancy may lead to increased 5-HT synthesis in the placenta and output to the fetus, resulting in abnormal serotonergic axon outgrowth into the developing forebrain.

It is therefore evident that chronic placental hypoxia and inflammation affect the catabolism of tryptophan in the placenta. It is suggested that IDO may act as a "sink" for superoxide, since IDO is known to utilize the superoxide anion as well as molecular oxygen for its oxygenase activity (93). A decrease in IDO expression as a result of hypoxia may therefore lead to decreased clearance of superoxide and an inflammatory response, potentially increasing placental 5-HT synthesis, with consequences for brain growth (94). Alternatively, decreased kynurenine synthesis as a result of hypoxia may shift the tryptophan catabolism pathway in favor of 5-HT synthesis. Clearly, the full effects of IUGR and placental hypoxia on placental tryptophan catabolism are largely unknown but likely to be important for setting the chemical environment in the IUGR brain, and determining vulnerability to damage arising from hypoxia, oxidative stress, or inflammation.

Inflammation and Neuroinflammation

The term inflammation is used to describe the production of cytokines, chemokines, reactive oxygen species, and secondary messengers, together with the paracrine and autocrine effects of these factors. In the CNS, microglia and astrocytes are the primary drivers of inflammation, i.e., neuroinflammation. Both systemic inflammation and neuroinflammation play a central role in the pathophysiology of various forms of perinatal brain damage, as shown by observations from both animal models and the human neonate (95–97). The levels of circulating cytokines in neonates born after IUGR are significantly increased on postnatal days 7 and 14 compared to levels measured in neonates without IUGR (98–100). This postnatal systemic pro-inflammatory state following IUGR could be, at least in part, responsible for the frank brain damage and neurodevelopmental impairments detected in childhood in these individuals. Indeed, at the heart of the vulnerability of the immature brain lies the systemic up-regulation of pro-inflammatory cytokines and the diffuse activation of cerebral microglia, the mediators of brain inflammation (95). The activation of microglia and astrocytes occurs via inflammatory signals coming from the systemic circulation via receptors on endothelial cells and the vagal nerve, and also by local pathogen- and damage-associated proteins (PAMPS and DAMPS, respectively) [reviewed in Carty and Bowie (101)]. In the context of IUGR, and in the absence of obvious pathogens, inflammation can come from at least two sources: (i) inflammation propagating from the placenta owing to the release of DAMPS due to tissue injury (see paragraph below), aberrant macrophage activation (102), and idiopathic villitis (103), and; (ii) from direct effects of hypoxia or other nutrient deprivation or intoxication on the brain (39–42).

Brain cell death, as a predicted consequence of hypoxia and other deprivations on the brain, would cause the release of DAMPS, such as HMGB1 (high mobility group box 1) that activate immune cells including microglia. Neuroinflammation, mediated by microglia, perturbs normal brain development directly by causing injury to cells such as maturing oligodendrocytes. In addition, an important set of developmental processes fulfilled by microglia is left undone when microglia are recruited to a neuroinflammatory response and this also damages the developing brain (see developmental dysfunction, below for further information on the role of microglia). A study of gene expression in microglia and

oligodendrocytes in a model of protein restriction-induced IUGR has revealed a striking induction of inflammation-related genes in microglia accompanying the reduction in oligodendrocyte maturation and connectivity and functional deficits (59). This was the first comprehensive study to link protein-restriction with neuroinflammatory-associated brain injury, and in the future, we will look for similarities in related animal models, and undertake human post-mortem studies to look for these dysregulated pathways. We also point the reader toward two excellent reviews on the role of microglia/neuroinflammation in IUGR, one written by a team of experts in pre-clinical modeling and brain injury including observations of microglia and macro-gliosis in various animal models, Wixey et al. (104), and one from the perspective of experienced reproductive immunologists with a focus on maternal immune activation as a driver of microglial activation, Prins et al. (105).

It is worth noting that neuroinflammation (i.e., activated microglia) typically carries assumptions of completely maladaptive or damaging processes. However, less well-understood processes of protection, repair and regeneration are also mediated by micro- and macro-glia, which occur at specific times after injury or insult (106–109). Although there has not been sufficient study of microglial phenotypes in clinical or preclinical IUGR models, more knowledge on microglial activation (phenotypes and temporal regulation) will likely help the development of drugs and treatments that exploit and expand the reparative effects of microglia, while decreasing the negative effects of these cells. Harnessing microglia to regenerate the brain is an approach being applied in the field of multiple sclerosis and adult neurodegeneration (110–112), and it clearly has a place in neonatal neurology.

Developmental Disturbance

The trajectory of brain development is altered by the presence of damaging stimuli, but also by the loss of cells, processes and factors that are important for brain building. As mentioned above, the causes and effects of IUGR include processes of inflammation, placental growth factor deprivation and hypoxia, which together affect the trophic actions of secreted factors such as serotonin, allopregnanolone, tryptophan, and IGF1/2. In addition, we wish to highlight that the "distraction" of microglia away from their normal physiological role in promoting proliferation, pathfinding, myelination and synaptogenesis will cause significant damage to the developmental trajectory of the brain [reviewed extensively in Prins et al. (105), Hagberg et al. (113), Pierre et al. (114) and Tay et al. (115)]

MANAGING THE RISKS OF CONTINUING PREGNANCY VS. PRETERM BIRTH—THE PRENATAL CARE TEAM

As there are currently no effective medical interventions for IUGR, management consists of close surveillance aimed at determining the most appropriate time for delivery. The definition of "most appropriate time" is a question for which the prenatal team has few specific criteria, and balancing the risks of prematurity with the consequences of IUGR (including, stillbirth) remains a contentious issue. Clinical trials to determine the optimal time of delivery have focused on survival and immediate perinatal outcomes, and often lack long-term follow up or an exploration of what features of delivery result in optimal long-term outcomes and reduced neurodevelopmental complications. Recent randomized controlled trials (e.g., Growth Restriction Intervention Trial [GRIT]; Trial of Randomized Umbilical and Fetal Flow in Europe [TRUFFLE]) have helped to shed light on delivery parameters of IUGR babies that lead to improved long-term outcomes. Both studies had a primary outcome of neurodevelopmental delay at 2 years of age.

The GRIT study randomized women at 24–36 weeks PCA to early or delayed delivery and included patients when the clinician was in equipoise about whether they needed delivery. "A priori" parameters set to determine when patients would be delivered were not used in this study. This agnostic approach can be seen as a benefit, as retrospective analysis could shed light on novel parameters common to infants who did well, but it is also difficult, as the motivation for the clinician's decisions are not easy to describe or document, and may not have been adequately captured in the analysis. There was generally only a 4-day delivery interval delay between randomizing the participants to immediate vs. delayed delivery. The 2- and 7 year follow-ups did not show a difference in neurodevelopmental outcome between groups (discussed further below)

The TRUFFLE trial was performed to examine whether parameters set "a priori" for delivery are effective in optimizing delivery outcomes. Participants with IUGR diagnosed at 26–32 weeks PCA with an elevated umbilical artery pulsatility index were randomized to delivery based on: (1) reduced cardiotocography fetal heart rate short-term variation (STV-CTG); or (2) early ductus venosus (DV) changes via doppler; or (3) late ductus venosus changes. They found that more infants randomly assigned to delivery based on late changes in the ductus venosus (95%) were free of neurological impairment compared to those assigned to cardiotocography (85%), but this was accompanied by a non-significant increase in perinatal and infant mortality. They therefore concluded that delivery based on late changes in DV flow might reduce long-term neuro-impairment. When the actual criteria for delivery in these cohorts was dissected it became clear that the majority of patients in the delivery for DV changes were being delivered based on the safety net criteria of spontaneous decelerations in the fetal heart rate. When the two cohorts being delivered for DV changes were combined the neurodevelopmental outcomes at 2 years of age were more favorable compared to those delivered based on STV CTG changes. Therefore, optimal neurodevelopmental outcomes may result if delivery of very preterm patients is restricted until late changes arise in the DV with a caveat of delivery whenever the CTG is abnormal (116, 117).

Severe growth restriction at term is also associated with poor neurodevelopmental outcomes (118). The Disproportionate Intrauterine Growth Intervention Trial At Term (DIGITAT) assessed the impact of immediate delivery vs. expectant management in patients with growth-restricted fetuses at term. The follow-up at 2 years of age demonstrated similar

neurodevelopmental gains. However, when assessed by gestation at birth, those in the lowest 2.3 percentile had significantly more neurodevelopmental compromise on an "ages and stages" questionnaire compared to those with a higher birth weight centile. Indeed, 43% of babies born with a birth weight centile below 2.3 had an abnormality on the their "ages and stages" questionnaire compared to 29% of those born at <10th centile and 13% of those with a birth weight greater than the 10th centile. This is consistent with a number of cohort studies demonstrating that low birth weight resulted in increased learning difficulties, defects in speech, neurological deficits, and behavioral problems (119).

BRAIN INJURY IN THE PRETERM BORN IUGR INFANT—THE POSTNATAL CARE TEAM

The incidence of spontaneous preterm birth in pregnancies with severe IUGR is 2 to 3-fold greater than the incidence of pregnancies with appropriate fetal growth (120). In addition, antenatal care focuses heavily on fetal growth monitoring in order to identify pregnancies with poor growth that may benefit from a timely, planned preterm delivery to improve outcomes. However, the contribution of antenatal compromise vs. the postnatal complication of being born preterm, or potential interactions between the two, in contributing to the neurodevelopmental sequelae of IUGR is still unclear. This is important for the clinical decision on when to deliver, when IUGR is diagnosed antenatally.

In a large cohort study comparing more than 1,400 preterm IUGR infants with age-matched AGA controls from 25 to 32 weeks PCA, the incidence of severe intraventricular hemorrhage (IVH) in each gestational age group was similar, but prematurely born IUGR neonates had increased morbidity and mortality (121). On the other hand, preterm birth has been suggested to override the effects of IUGR *per se* on neurological outcomes (122, 123), with the impact most marked for births at the earlier gestational ages. In early-onset IUGR, the gestational age at delivery shows a consistent independent relationship with parameters of motor development with a maximum impact for infants delivered before 28 weeks PCA (124–126), independent of the severity of attrition of growth and the degree of cardiovascular and biophysical deterioration. In the GRIT study which randomly allocated women to early or delayed delivery in the presence of IUGR, and when the obstetrician was unsure whether to deliver, 98% of these patients (n = 376) completed a 2 year follow-up, revealing that the rate of cerebral palsy was greater for patients delivered prior to 31 weeks PCA, and prematurity-related complications were important contributors to this risk (127). Notably, the relationship between gestational age, IUGR and motor deficits suggests the impact of IUGR becomes more apparent with delivery at a later gestation (128). The large prospective EPIPAGE (Etude EPIdémiologique sur les Petits Ages Gestationnels, epidemiologic study of early gestation ages) study (>5,000 births) examined neurological outcomes in school-age children that were born AGA or IUGR (<10th centile for birth weight) at 24–28 weeks or 29–32 weeks PCA, and found similar cognitive deficits in AGA and IUGR infants born at 24–28 weeks PCA, but much less in AGA infants born at 29–32 weeks (129). This clearly indicates the importance of *in utero* brain maturation up to at least 32 weeks PCA. The rate of neurocognitive deficits in the moderately preterm infants with IUGR was around 40% and was identical to the incidence of neurocognitive deficits in extremely preterm infants (129), indicating that the impact of intra-uterine and extra-uterine adverse conditions may be similar on the developing brain in early third trimester. However, a limitation of the EPIPAGE study was stratification of infants by birth weight alone, with no supporting evidence for IUGR.

Consistent with these findings, another study using neuroimaging showed that at 6 years of age, both extremely preterm infants (born before 28 weeks) and moderately preterm IUGR infants (born after 28 weeks), had decreased brain connectivity (measured using MRI fractional anisotropy) when compared with moderately preterm AGA controls, which in turn is associated with poorer socio-cognitive performance (130). IUGR infants born moderately preterm and assessed at term equivalent age also demonstrated reduced cerebral cortex gray matter volume and lower scores in attention-interaction availability, compared to appropriately grown preterm infants (131). Again, these observations support the importance of *in utero* brain maturation.

Interestingly, the rates of several prematurity-associated neonatal diseases in IUGR vs. AGA infants also vary with the gestational age at birth. In preterm infants born at or before 28 weeks PCA, the rates of IVH, respiratory distress syndrome and necrotizing enterocolitis are largely unaffected by IUGR. From then on, all adverse outcomes including IVH increase in IUGR compared with AGA premature infants, suggesting a need for closer surveillance for IUGR in the moderate and late preterm infants (118, 132). Such findings are of concern since late preterm births account for the vast majority of preterm births. Studies on infants born at later preterm to term gestational ages suggest that impaired fetal growth increases the risk for low intellectual performance (16, 133, 134). Comparing monozygotic twin pairs born after 32 weeks PCA, the growth-restricted twin is at increased risk for low cognitive performance at school age or in adulthood compared to the appropriately grown twin (135). In addition, a further study in twins has quantified the effects of low birth weight, showing that a 500 g increase in (term) birth weight results in a 2% increase in total brain volume, gray matter volume and white matter volume, and a 2-point increase in IQ (136).

In summary, the gestational age at delivery has a remarkable impact for IUGR infants who are born extremely preterm, such that the prematurity-related complications "override" the effects of IUGR on neurodevelopmental outcome. For the moderately late preterm infants, the independent impacts of IUGR and prematurity on neurodevelopmental outcome becomes more apparent. Altogether, these studies highlight that we need more information on when to deliver IUGR babies, and the criteria on which to base this decision.

CLINICAL TRIALS AND PRESUMPTIVE THERAPIES

We wish to acknowledge the enormous contributions of researchers and clinicians in bringing therapies to clinical trials and generating the preclinical data to support these transitions, although the purpose of this article is not to review all of these. We will highlight some trials and their outcomes as, while we have no conclusively effective therapies as yet, we can learn a great deal about improving patient stratification and the efficacy in drugs with shared mechanisms of actions. For example, a recent double-blind randomized study using dydrogesterone, a synthetic progestogen, has reported increased birth weight, and decreased MCA resistance index in idiopathic IUGR (137), supporting the therapeutic use of progesterone replacement suggested in preclinical studies in guinea pigs (138). Specifically, in this human trial weight increased by ∼50% in the treatment arm, vs. 23% in the control arm. Although these effects are promising, this was a single center, small study (89 participants), recruiting early and late IUGR (range 28–35 weeks of gestation) with no postnatal follow-up, thus further work remains to be done. The action of dydrogesterone includes effects that altogether increase myometrial perfusion and immunomodulation by increasing progesterone-induced blocking factor (PIBF) levels (139). PIBF is secreted by peripheral lymphocytes from healthy pregnant women, and it has important immunomodulatory functions that appear to protect fetuses from resorption and therefore plays a role in the maintenance of pregnancy, most likely by inhibiting NK lymphocytes and producing a dominant TH_2 cytokine response (140); hence, many poor pregnancy outcomes including miscarriage and preterm birth are linked to low PIBF levels. Manipulation and control of PIGF levels in pregnancy is therefore a priority for researchers.

The STRIDER-UK study (multicentre, randomized, double-blind, 156 participants) tested the use of sildenafil in women with severe early-onset IUGR and found that treatment did not prolong pregnancy or cause any adverse effects, but did not improve pregnancy outcomes (141). However, this study is part of a more extensive international study, and in isolation, it does not have the power to adequately assess outcomes in this very high-risk cohort (45% of recruited infants in this study died) (142). A meta-analysis (nine studies, total of 576 treated patients) has shown that arginine supplementation increases gestational length and birth weight in IUGR pregnancies, except for infants born preterm (<32 weeks PCA) with severe IUGR (143); there were no reported side effects. The proposed mechanisms of action of arginine include the increased production of placental insulin that acts as a fetal trophic factor.

Creatine may also be a potential treatment for IUGR (144, 145), with recent studies showing a positive correlation of birth weight to placental creatine load (146), and the discovery that the human placenta expresses the enzymes to synthesize and transport creatine (147). Creatine is an energy substrate which protects ATP turnover during periods of oxidative stress (148) and as such may be a potential prophylactic treatment for IUGR outcomes (144, 145). Creatine readily crosses the placenta in humans and some other omnivores (but not in sheep, an herbivore), suggesting that maternal creatine supplementation could be used to increase placental creatine transfer and promote fetal growth in a hypoxic uterine environment. Supporting data include a number of pre-clinical studies (149–151). Whilst there has been extensive animal research suggesting creatine's potential to protect the fetus against periods of oxygen deprivation in several animal models, and there is a strong rationale for moving toward clinical trials for maternal creatine supplementation to reduce or prevent IUGR, no intervention studies have yet been undertaken in pregnant women. IUGR is also proposed to be a disorder of insulin-like growth factor-1 (IGF-1) deprivation (discussed below). Of particular note is an extensive preclinical study of prenatal IGF-1 treatment in a sheep model of IUGR (41 controls, 66 IUGR + saline, 28 IUGR + IGF-1, powered for sex-specific analysis and long term follow-up) which show that prenatal IGF-1 treatment improves prenatal and postnatal indices of growth and biochemical dysfunction in IUGR (152, 153).

OVERCOMING THE HURDLES TO PROGRESS

Early Markers for IUGR Screening: A Challenge Faced by Researchers and Clinicians

Less than 30% of infants with a birth weight <10th percentile are detected during pregnancy (154, 155). Infants born after undiagnosed IUGR have 2–9 times higher risk of perinatal death and severe neurological complications to those diagnosed prenatally (156–158).

The current, commonly used antenatal assessment of IUGR is examination of the symphysiofundal height, and this has a sensitivity of just 17% and a positive predictive value of 20% (159). Once a red flag has been raised, even selective ultrasound and universal ultrasound perform poorly with a sensitively of 20 and 57%, respectively (160). This perhaps reflects the larger caliber vessels and reduced downstream resistance of the late preterm placenta that means that the umbilical artery Doppler parameters are rarely abnormal; indeed, most adverse events in late pregnancy occur in fetuses with normal Doppler readings (161). However, as mentioned above, FGR babies assessed at term age will often demonstrate abnormal middle cerebral artery (MCA) blood flow represented by a reduced MCA pulsatility index (PI) due to "brain sparing." A more sensitive and specific measure of fetal well-being may be the cerebral-placental PI ratio, which is calculated as the MCA PI divided by umbilical artery PI. This parameter has improved the sensitivity for detecting babies at risk of adverse perinatal outcome including perinatal mortality, admission to NICU, low 5-min Apgar score and cesarean for fetal distress (26, 162, 163).

Given the importance of placental insufficiency for the origin of IUGR, blood-based markers in the mother that relate to early-onset placental insufficiency are logical starting points for identifying biomarkers that detect IUGR. Biomarkers are molecules, genes, or a particular combination of these by

which a pathological or physiological process is identified. Recent reports of biomarkers in maternal blood related to early-onset placental insufficiency include pregnancy-associated plasma protein-A (PAPP-A), alpha-fetoprotein (AFP), inhibin A, placental growth factor (PlGF), uric acid, and free beta- or total human chorionic gonadotropin (102, 164). For example, a 2 to 3-fold increase in late-onset IUGR is noted in women who were found to have elevated first trimester PAPP-A and elevated second trimester AFP (165). Combining maternal risk factors, biochemical markers such as ADAM12 (A Disintegrin and Metalloprotease-12) and placental protein-13, plus abnormal uterine artery waveforms for the prediction of late-onset IUGR (with a false positive rate of 5%), provides a sensitivity of 61% for IUGR at <37 weeks but unfortunately only 32% for IUGR at >37 weeks (166).

The use of these putative biomarkers (and the development of new biomarkers) is constrained by the absence of robust baseline data on their expression and function. We could overcome these problems with population profiling and further basic research into the mechanisms underlying typical placental development and function. Biomarkers for identification and stratification could improve outcomes even with current clinical practices, and are likely to have two additional benefits: firstly, prenatal treatment options [e.g., intra-amniotic IGF-1–(153)] are showing great promise, and biomarkers would identify those needing therapy; and secondly, by avoiding treatment of healthy babies and the risk of complications arising from newly developed prenatal therapies.

In utero assessment of brain structure and metabolism is now possible, and these techniques have been applied to cohorts of IUGR infants (167). These studies aim to understand the underlying pathology, but also to determine biomarkers as indicators of pathology and outcome. For example, in 19 IUGR and 25 non-complicated pregnancies, T2-weighted MRI allowed for the assessment of neuropathology and magnetic resonance spectroscopy allowed for analysis of indices of cell membrane and myelin formation (choline), glycolytic enzyme activity as an indicator of cerebral hypoxia (lactate), and for cerebral mitochondrial function (NAA) (167). Of the 15 infants with complete data sets in the IUGR arm, three died *in utero*, and only two had an uncomplicated neonatal course. However, MRI was not sensitive enough to detect injury in any of the 15 IUGR infants *before delivery* (median scan age of 27 + 6 weeks PCA). Interestingly, fetal brain lactate levels were elevated in three control infants with normal progress of growth, birth and outcomes. Increased brain lactate has previously been considered a hallmark of hypoxic changes, but lactate may also play a role as an energy source in the developing brain (168). The authors suggest that the altered magnetic resonance spectroscopy parameters represent changes in mitochondrial metabolic status, and these warrants further study in a preclinical model as a novel therapeutic approach. This study is a valuable example of how these still tricky and expensive prenatal screening techniques could be applied to larger cohorts, but whether this effort can provide invaluable data on the mechanism of damage and criteria for risk stratification will require further research.

Circulatory biomarkers based on the detection of microRNAs (miRs), mitochondrial DNA (mDNA), cell-free RNA (cfRNA), and exosomes could also have diagnostic value, but remain to be fully validated. Two recent studies demonstrate how these new analytic approaches provide early diagnostic biomarkers for preterm birth and preeclampsia. Firstly (169), demonstrated that a panel of seven cfRNAs present in the maternal blood had utility in predicting preterm birth with an AUC (area under the curve) of 0.81. Similarly, Jelliffe-Pawlowski et al. (170) used a 25-target screen of serum proteins that, together with maternal risk factors, predicted preterm birth with an AUC of 0.806. Both studies might be said to be limited by low patient numbers and a lack of ethnic diversity in the patient groups, but this multi-marker approach seems to increase sensitivity and specificity compared to previous mono-marker approaches, and improvements (decrease) in the time needed to perform such analyses means that effective bedside screening is becoming a reality.

A further elaboration on the multi-marker approach is the application of personalized risk-based screening methods that combines maternal factors and biomarkers, as in the combined use of uterine artery Doppler, maternal risk factors, and serum biomarkers. This approach, taken as part of a study nested in clinical trial for pre-term preeclampsia (ASPRE, Aspirin for Evidence-Based Preeclampsia Prevention) (171), showed clearly that prospective screening for preterm preeclampsia by means of the FMF (Fetal Medicine Foundation) algorithm, which combines maternal factors and biomarkers at 11–13 weeks' gestation, was better at predicting disease risk than current biometric criteria.

A Holistic vs. Mechanistic View of Modeling IUGR—How to Improve the Validity of Our Models

An important question when modeling any disease or disorder is whether it is necessary for the phenotype (in this case a reduction in body and brain weight) to be the same as observed clinically if we don't know that the mechanism of injury has been faithfully reproduced. Also, how much importance should we place on the phenotype (body and brain weight) being matched if we have to apply an injury/perturbation (in type or magnitude) that is not clinically relevant? In the case of IUGR there has been a proliferation of animal models that produce fetal growth restriction, but often by means that have limited clinical reality; e.g., abrupt and late onset reductions of uterine or umbilical blood flows. These procedures do produce hypoxia-induced cell death, and various complex inflammatory processes that help us to understand and look for effects in IUGR infants, but they are of limited use in understanding how IUGR and placental dysfunction actually arise in human pregnancy. Perhaps, with our increasingly detailed knowledge of how and when placental dysfunction in IUGR occurs (88, 172, 173), we could aim to model these specific changes, and this would be valuable in the collective move toward developing therapies that can be applied early in pregnancy. We will below describe some of the common approaches to modeling IUGR and some suggestions for how

these could be altered to add to our collective data on how to prevent IUGR, and treat or repair the damage caused by IUGR.

Surgical interventions that reduce perfusion of the maternal or fetal side of the placenta: The seminal study demonstrating that poor placental growth itself causes attrition of fetal growth is that of Wigglesworth (174). Since that time there has been a proliferation of models of IUGR, predominantly focused on fetal/neonatal weight and based on the conceptual paradigm that it is utero-placental perfusion that determines placental function, which in turn determines fetal growth and the vulnerability of the fetal brain to damage. These models of IUGR include (but are not limited to): acute onset hypoxia/ischemia (uterine artery ligation), progressive onset hypoxia/ischemia (uterine artery restriction); and placental damage/reduction (inert microsphere injection, partial placentotomy). However, these approaches offers little consideration to the idea that the fetus(es), or the feto-placental compartment might themselves be the source of physiological changes that cause the typical uterine hyperaemia and the appropriate increase of utero-placental perfusion with increasing gestation. Evidence of this type (and almost forgotten) was acquired nearly 50 years ago by Christopher Bell in guinea pigs who showed that progesterone produced by the placenta causes a loss of constrictor adrenergic nerves in uterine blood vessels, and simultaneously induces synthesis of a vasodilator mechanism not present in the non-pregnant uterine vasculature (175, 176). Hence, there is a good reason to think that a cause of IUGR is the *failure* of the placenta to adequately modify the uterine circulation to support time-dependent fetal growth. As such, more work needs to be done on the differences in feto-placental signaling as a driver for poor utero-placental vascular development across gestation.

In all experimental studies to date, the attention is almost always directed to the fetal effects, with few observations made of maternal or placental physiology, especially the clinical conditions usually associated with IUGR, such as uterine artery and myometrial remodeling, immune regulation of trophoblast implantation, placental metabolism *per se*, and the causes of placenta infarction and abruption, etc. A chief mechanism by which we should validate animal models of IUGR should be to measure uterine blood flow, as is done clinically. Admittedly, historically this was not easy to do in rodents, although it is increasingly possible due to advancements such as ultrafast doppler. We now have the ability to measure these clinical indices and to associate them with fetal outcomes as we attempt to better understand this relationship.

Poor placental structure and function—from the beginning: Placental insufficiency is a process that evidence suggests is present from the earliest stages of trophoblast invasion, supported by the observation of abnormal levels of inflammatory and placental factors from the 1st trimester with poor pregnancy outcomes [(47) and reviewed in Kane et al. (177)]. From the time of trophoblast invasion into the endometrium, the uterus undergoes phenomenal modification (178, 179), increasing in weight and internal volume by ∼16- and 500-fold, respectively, together with major re-modeling of the uterine vasculature (as discussed above) to create a low resistance, non-reactive vascular bed. It is perhaps not surprising that these features have received less attention as determinants of IUGR because, except for non-human primates, these key features of pregnancy are not present in the commonly used laboratory animals. However, comparative studies of more unusual species, like the Spiny Mouse (*Accomys caihrinus*) reveal that unlike in the conventional rodent that there is a large vascular contribution to the fetal membranes originating from the umbilical vessels close to the fetal surface of the placenta (unpublished observations). As such, studying basic process of placentation in small species such as these may be a useful start to developing models of early-onset placental dysfunction that mimics what is seen clinically (57, 152, 180). Non-traditional species such as the spiny mouse, gerbil, and guinea pig studied from the time of conception offer advantages such as a relatively long gestation (from 39 to ∼67 days), and the birth of offspring where development of the major organ systems is largely complete at the time of birth. For the spiny mouse in particular, an additional advantage is that the fetal adrenal gland produces dehydroepiandosterone and cortisol (181–183), with evidence of the presence of a feto-placental unit as in humans, and not present in other rodent-like animals, or sheep.

A clear example of how a candidate mechanism has been effectively applied to model IUGR is the knockout of IGF2 production in the mouse placenta. Levels of the IGF proteins are often reduced and IGF1 binding proteins increased in the placentas of IUGR human pregnancies (184), although this is not consistently found (185). The regulation of IGF signaling is a complex balance between the IGFs and their binding partners that regulate bioavailability during pregnancy, including in IUGR [reviewed in Martin-Estal et al. (186)]. Mice born from dams with a placenta-specific IGF2 gene knock out (KO) have clearly reduced body weight, brain injury, and lasting cognitive and metabolic phenotypes with postnatal development. The mating of the IGF2 placenta-specific KO mice with the endothelial NOS (eNOS) KO mice provides an interesting example of the cross-talk between (putative) pathological mechanisms. As expected, the phenotype of these two KOs was additive (more severe IUGR), but the diminished placental nutrient transport typically observed in the eNOS KO mice was not seen in the IGF2-eNOS double crossed mice (187). The basis of the application of the eNOS KO mice to IUGR research is that the absence of eNOS reduces the capacity of the maternal vascular to accommodate the changes in blood flow necessary for adequate placentation, and that the dams are hypertensive (including with proteinuria) as observed in women with pre-eclampsia, a risk factor for IUGR. Thus, it was concluded that a "multiplicity of dysfunction" probably underlies IUGR in women, so the multiple facets of vascular dysfunction in this mouse line would be useful to assess placental hypoxia with free radical formation, reduced placental nutrient transport capacity, and reduced fetal growth (188).

Assessment of the epigenetic landscape of the eNOS and IGF2 genes from IUGR placental tissues shows that epigenetic modifications are present and might be drivers of gene dysregulation (189). However IGF2 gene expression changes and methylation changes of IGF2 are not consistently reported in clinical studies, One of the negative clinical studies found neither gene or methylation changes the cohort was older mothers (39–40 years of age), although changes in placental methylation

are only detectable with the techniques used when substantial differences are present (173). Alternatively, this negative study is evidence that methylation and gene expression changes in IGF2 are not present in the first trimester but evolve over time, and so this important study that warrants repeating with larger and more diverse cohorts.

Immunological dysfunction: There is clear evidence that the immune system in women with an IUGR pregnancy differs compared to those without IUGR (190). This study found that peripheral monocytes in women with an IUGR pregnancy had a more classically anti-inflammatory profile than monocytes from women with an uncompromised pregnancy. In addition, when peripheral blood mononuclear cells from women with IUGR and normal pregnancies were stimulated with trophoblast antigen (191), a greater pro-inflammatory reaction occurred in women with an IUGR pregnancy. When these dysfunctional processes first arise in pregnancy, and their impact on early placentation, would be interesting to pursue further, albeit requiring large cohorts of prospectively recruited, not-yet-pregnant women—a difficult task, but one with important public health outcomes. This information would then be possible to overlay into more specifically focussed animal models.

As pointed out by Sir Peter Medawar many years ago (192) it is clear that immune modulation is a fundamental response to conception that allows implantation and persistence of the fetus as a foreign allograft, and a response likely to be shared by humans and laboratory animals. It is a process that involves a phenotypic transformation of decidual macrophages and natural killer cells that occurs in parallel with remodeling of the vasculature adjacent to the implantation site. As such, it is possible that these shared features could be used to drive spontaneous cases of IUGR in non-human pregnancies. Indeed, some cross-breeding of mice of mixed genetic background has been found to produce IUGR of varying degrees, together with, as for most clinical situations, other complications of implantation, uterine re-modeling, and pathophysiology suggesting preeclampsia in the dam (193, 194). These "immune" models have been shown to have similarities pathological changes as occurs in the human IUGR placenta in the trophoblast remodeling protein Formyl peptide receptor-2 [FPR2; (195)], and a dysfunction of decidual arteriolar remodeling (196) such as that associated with pre-eclampsia and IUGR (197). Thus, we may have experimental models at hand which will aid in the understanding of the origin of IUGR together with the other obstetric problems that usually accompany it. A strength of this approach is that each of the parents and the offspring are immunologically competent, and it is only the combination of the fetal and maternal tissues that is abnormal. This model will require further assessment of fetal brain phenotypes to support the similarities in placental immune dysfunction to the human, but it promises to be valuable to assess therapies to minimize the neonatal morbidities that IUGR produces.

Finally, we would like to highlight the comments made by Saleem et al. from Karachi, Pakistan that "Concerted efforts should be made to gather indigenous data about the risk factors of IUGR that are more pertinent to our population. Evidence-based recommendations deduced from such data sets are more likely to be successful and valid" (198). Although they were addressing the need for improved medical services, we think this comment also points to the need for improvements in preclinical modeling to be "*population specific.*" This specificity must take into account not just high vs. low income, but etiologies and conditions specific to local care centers, and this is where in-depth epidemiology needs to take a more prominent role in guiding preclinical research.

Human Neuropathological Studies and How They Need to Play a Bigger Role in the Study of IUGR

The emergence of modern medicine was firmly based on pathology and post-mortem examinations, and while never being able to identify mechanisms of disease or injury *per se*, have been invaluable as the basis for setting diagnostic criteria and the effectiveness of treatments and interventions. The expectations of many that modern imaging, genetics, and -omics would make pathology obsolete has been a significant error of twenty-first-century science, and in neonatology it was high-quality neuropathology studies by groups in Portland (OR, USA), Paris (France), and Boston (MA, USA) (199–201) that were persuasive in changing our understanding of the genesis of perinatal brain injury over the past 20 years. Specifically, these foundational studies provide the basis for interpreting MRI studies, and for designing improved animal models to test neuroprotective strategies. This is important because most IUGR babies survive, and the injuries present in very severe IUGR fetuses may not be representative of the major population of IUGR infants.

It is indeed worth highlighting a number of human studies; this is not exhaustive but is intended to allow us to remark on what we have learnt, and what we need to add to the field. Studies from Samuelsen et al. (202) show a reduction in the numbers of cells in the brains of IUGR infants; these infants displayed brain sparing (relative increase in brain weight to body weight) but still had significantly lower brain weights. In this study, control fetuses acquired an average of 173 million cells per day from mid-gestation to term, and the IUGR fetuses acquired only 86 million new cells per day. Samuelsen et al's exhaustive cell counting assessment [reviewed in, Larsen (203)] supports the findings of two studies from over 30 years ago which used a lower total DNA content to infer a reduction in cell numbers (50, 204). Samuelsen's conjectures that his data supports the hypothesis that the reduction in head circumference in IUGR is due to reduced proliferation rather than cell death is provocative, but needs to be confirmed by objective assessment of cell death and proliferation.

Another set of studies includes a concerted effort by researchers based primarily at the Mater Hospitals in Brisbane, Australia, that included 37 asymmetric IUGR cases (weight <3rd centile) identified from a series of 225 stillbirths (205). This first study, over 20 years old now, critically demonstrated the importance of cell death, and to a lesser extent astrogliosis, in the IUGR brain. It is worth noting that although the analysis was limited to hematoxylin and eosin assessment of cell death, and in some cases glial fibrillary acidic protein for astrocytosis, that even with the expertise of a neuropathologist no injury was found in 5 of the 37 brains. Of these 5 "uninjured" IUGR

brains, four were from infants below 26 weeks PCA—does this suggest an important window for treatment specificity? Striking limitations of this study are that there was no breakdown of the specific gestational ages of the infants, no body or brain weight data, and no case-by-case description of the findings, making it very difficult to draw further conclusions. These authors overcame many of these limitations in a more recent publication (206) using brains from the first cohorts and sourced from a further 305 stillbirths where they assessed cell death with three separate markers. This more recent study clearly showed that third trimester stillborn fetuses with both IUGR (weight <3rd centile) and placental infarction (<5% of total placental villus surface) had neuronal apoptotic changes in regions including the pons and the frontal and temporal cortex. These studies involved the use of controls that were stillborn but had neither IUGR nor placental infarction. Staining for micro- or macrogliosis or proliferation was not included, unfortunately.

The Value of Continuous Neuro-Monitoring to Understand Risk for the IUGR Infant

Much has already been reported on the neuroimaging findings of IUGR infants and their relationships to neurodevelopmental delay. However, neuroimaging such as MRI can only be done at specific time-points and is not applicable as continuous neuro-monitoring to reveal temporal changes with impact on clinical conditions and to guide management.

Cerebral Haemodynamic Measurements in the IUGR Fetus

With advancing fetal hypoxia and compromise, the cerebral haemodynamic response involves two components—firstly an initial stage of increased cerebral blood flow aimed at protecting the brain (brain sparing), followed by a second decompensatory stage that is associated with brain injury, and probably due to the increased cerebral blood volume (207). At the early stages (i.e., brain sparing) blood flow (CBF) of the frontal lobes is increased, perhaps with the effect of protecting higher cognitive functions, but under chronic and more severe circumstances this change is lost and CBF is diverted to deeper (more essential?) structures such as the basal ganglia and the brainstem (29). Also, in the IUGR fetus there appears to be a loss of cerebral vasoreactivity; using prenatal Doppler sonography, a subset of IUGR fetuses did not show the expected rise in cerebral resistance in response to maternal hyperoxygenation, suggestive of impaired cerebrovascular regulation. Indeed, these "non-responders" had a higher risk of being delivered for fetal distress, indicating that they were more compromised (208).

It is interesting that there is an increased incidence of stroke in adults born with low birth weight. The association between low birth weight and adult stroke was most pronounced for individuals with relatively increased head size, suggestive of *in utero* conditions that induce brain sparing (13). It is very plausible that these mechanisms of increased adult stroke are due to vascular remodeling secondary to the shear stress and wall tension, leading to structural changes in the vascular wall (209). Thus. understanding IUGR-associated cerebral haemodynamic changes has implications for improving brain health even in adults.

Cotside Cerebral Haemodynamic Measurements: Cerebral Blood Flow and Oxygenation

Studies in IUGR infants have made the following important observations:

- increased CBF on the first day of life (210);
- reduced cerebrovascular resistance and persistent dilatation of the cerebral arteries (211, 212);
- higher regional cerebral oxygen saturation and reduced cerebral oxygen extraction within the first 24 h (213) and up to 3 days of age (214); and,
- cerebral haemodynamic parameters normalize within a few days of birth (210, 211, 213).

To date there is little postnatal research investigating the relationship between cerebral hemodynamics after birth and neurological injury in IUGR infants; this is an area of need as there is evidence to indicate that the altered cerebral hemodynamics that exist in the IUGR fetus persist postnatally. Notably, cerebral oxygenation has been shown to predict neurodevelopmental outcome in preterm infants (215). Moreover, fluctuations of cerebral oxygenation and oxygen extraction have also been related to the occurrence of intracranial hemorrhage in preterm infants (216, 217). Finally, if CBF remains elevated when the neonate is no longer exposed to a hypoxic environment, the increased CBF could cause hyperoxia within the fragile brain and contribute to further neurological damage (218).

Cotside Cerebral Haemodynamic Measurements: Cerebral Autoregulation

Autoregulation is the ability of the cerebral vasculature to maintain reasonably constant CBF despite fluctuations in cerebral perfusion pressure which are mainly affected by changes in systemic blood pressure, and it is likely that autoregulation is impaired in sick preterm neonates and this contributes to the cerebral ischaemic and haemorrhagic injury (219). As IUGR fetuses are often delivered preterm, and due to the vascular structural and functional changes set in place by the "brain sparing" response to IUGR, these infants are theoretically at risk of impaired autoregulation, resulting in cerebral hypo- or hyperperfusion when systemic blood pressure fluctuates. In case of prolonged brain sparing due to chronic hypoxaemia, maximal cerebral vessel dilatation may have already been reached and may persist after birth, which would further limit protective autoregulatory responses. In addition, IUGR neonates appear to have higher blood pressures compared to their AGA peers (30, 220), theoretically contributing to risk of cerebral hyperperfusion and hemorrhage in the presence of a pressure-passive cerebral circulation; indeed, this has been shown in IUGR lambs where, compromised structural integrity of the cerebral microvasculature leading to cerebral hemorrhage has been demonstrated (82). Despite its clinical significance, to date no study has investigated cerebral autoregulation in human IUGR neonates.

Cotside Electroencephalographic (EEG) Monitoring

Cerebral electrographic activity can serve as an indicator of neuronal integrity, organization, and the differentiation and maturation of brain networks in term and preterm newborns (221). In acute brain insult, electroencephalographic (EEG) activity shows various degrees of depression, and its severity parallels the magnitude of the brain lesion. These "acute-stage" abnormalities gradually improve with time and are replaced by "chronic-stage" abnormalities such as dysmaturity and disorganization of the EEG pattern (222). In the weeks following preterm birth, IUGR infants reportedly have altered EEG and amplitude integrated EEG, which has been correlated with poor neuromotor development (223, 224). In contrast, another study found that the subset of stable preterm IUGR with good clinical indices had more mature EEG patterns compared to the AGA peers (225), and similarly, preterm IUGR infants had accelerated EEG power spectrum maturation compared to preterm AGA controls at 1 month post-term equivalent age (226). However, no differences were observed at 6 months' post-term age between preterm IUGR and AGA infants, or in comparison to a term AGA group, suggesting such changes may resolve with time (226). Notably, using visual evoked potentials as an indicator of brain myelination in preterm infants born at <33 weeks of gestation age, shorter visual evoked potential latency, suggestive of increased myelination, was found at 6 months post-term age in IUGR infants who had fetal Doppler parameters showing brain sparing (227). The shorter latency was no longer detected at 12 months of post-term age and visual functioning was not affected when followed up at 11 years of age (228). These findings suggested transient accelerated neurophysiological maturation in the IUGR infant brain, possibly as an adaptive process to the severe fetal growth restriction. Overall, it appears there are temporal EEG characteristics in IUGR infants which may relate to their neurodevelopmental outcome. However, EEG data on preterm infants with IUGR are few, as are studies that link EEG parameters with MRI-derived tractography. Early and prolonged continuous recording in larger populations would be required to clarify the prognostic value of EEG in IUGR infants.

INNOVATIONS FOR USE IN IUGR RESEARCH:

Nanomedicine Approaches

Engineered nanomaterials offer therapeutic options as diverse as implantable monitoring devices, drug delivery scaffolds, and wound dressings. An excellent review on nanomaterials for perinatal applications was recently published (229), but we will highlight ways in which these approaches could be employed to improve infant neurological outcomes in the context of IUGR. The greatest need for the IUGR fetus is safe, effective therapies that can be delivered as early as possible in gestation and without the need for preterm delivery. As such, we should turn our attention to nanomedicine approaches if these can be maternally delivered and directly affect the function of the placenta. Nanoparticles delivered intravenously can cross the placenta, and are found in the fetus, including in the non-human primate [see Menezes et al. (230)]. They can also be found in the fetus when administered intranasally to pregnant rats (231). A recent study monitored the "protein corona" of polystyrene nanoparticles, which is the spontaneously adsorbed protein on the nanoparticle surface when the nanoparticles were exposed to maternal plasma. They found that the protein corona formed by maternal serum included vesicular transport proteins such as clathrin, tubulin, actin, and Ras. As such, rather than pose any barrier to transmigration, maternal serum proteins increase the transport of the polystyrene nanoparticles across the *ex vivo* placenta (232). However, in the pregnant rat, inhaled silver nanoparticles caused increased fetal resorption and increased expression of placental inflammatory markers (231). Although this silver nanoparticle study suggests caution in the application of nanoparticles, we can overcome these problems via our ability to modulate the size, physical composition, charge, and delivery method of nanoparticles. Of note, another recent study has shown success in reducing fetal inflammation and neonatal brain injury in a mouse model which mimics exposure to inflammation in the preterm infant. Specifically, polyamidoamine dendrimers were used to carry the anti-oxidant n-acetylcysteine into the blood of the dam (233). This dendrimer treatment was associated with improvements in inflammatory and neuropathology indices in the fetal compartments. It is important to note that these polyamidoamine dendrimers did not need to pass to the fetus to be neuroprotective and thus, this maternal-placental delivery reduces any potential of off-target, damaging effects of stray nanoparticles in the fetus.

Work from our lab has taken the approach of targeting one cell type—microglia—rather than being compartment-specific (234). Microglia are regularly found to mediate injury in neurological paradigms. The microglia-specific nanoparticles in our work are comprised of DNA, further demonstrating the flexibility in the design possibilities of nanoparticles. However, we do not yet know if these DNA nanoparticles cross the placenta, and what specific aspect of microglial activation would be the most appropriate to target in IUGR. There is a lack of knowledge of nanoparticle biodistribution in non-human primates, or other species with more human-like placental structures, such as the spiny mouse (235). More studies using IUGR models developed in species with a more human-like placental structure would be valuable steps toward uncovering the potential of nanoparticles to deliver anti-oxidants, vasodilators, or growth factors to specifically target pathological processes in the placenta.

Altogether these tools and approaches show that we have made the technological advances and have the monitoring skills with which to move forward to design targeted therapies for the placenta and the fetus to overcome the damage associated with IUGR.

Using Advanced Imaging and Histology Techniques

We have a poor understanding of the direct correspondence between medical imaging outputs and tissue microstructure. This lack of knowledge is due to differences in scale and resolution between medical imaging modalities and traditional

neuropathology techniques that have been further frustrated with complexities of image registration (236). A lack of knowledge of the actual biological substrate of imaging outputs frustrates our ability to understand the specific nature of changes occurring in the IUGR infant brain, as the most severely affected infants do not survive, as discussed above. Improvements in histological techniques, specifically the use of optically clear histology, is allowing us to overcome some of these hurdles (237), but obviously not in living tissue. Optically clear histology allows whole brains from small experimental animals, and sections of human post-mortem tissue, to be made optically clear and stained with multiple specific markers of various cell types. This advance allows us to visualize the 3D organization of tissue microstructure in tissue sections large enough to be studied beforehand with MRI techniques, and then to co-register the imaging and histological data. This approach has been applied to the developing mouse brain to provide data on what types of cells or cell compartments contribute to specific imaging signals (238). A finding from this study worth noting is a positive correlation between mean MRI diffusivity and cell density, which is unexpected based on the current assumption that cells provide an impediment to the diffusion of water molecules in tissue (238). This and other findings suggest that a great deal of work in human post-mortem tissues is required to ensure that our assumptions of the microstructural correlates of human imaging in the literature are robust (239, 240).

The current gold standard for functional brain imaging is blood–oxygen dependent (BOLD) signals measured using MRI (241), but this technique is costly and practically difficult to use in infants. An easier to implement optical imaging alternative is functional near-infrared spectroscopy (fNIRS), which also measures the level of blood oxygenation and can be applied on neonates or young children through the skull but with spatial resolution measured in mm (242). The next level of technological development is functional ultrasound (fUS) neuroimaging that provides high sensitivity imaging, with a resolution of $\sim$100 μm, and can identify cerebral blood volume changes in the whole brain without contrast agents. The fUS image is based on the phenomenon of neurovascular coupling, about which little is known in the developing brain [see, (243, 244) for recent animal studies] and the ability to measure cerebral blood volume changes with high sensitivity, which is an attribute of fUS (245). Non-invasive fUS imaging of brain activity in humans is possible through the fontanel of human neonates at the bedside. In combination with surface EEG recordings in preterm babies, fUS allowed for the estimation of cerebral blood volume variations to measure the spatiotemporal dynamics of epileptic seizures (246). The sensitivity of fUS, such as measuring small diameter blood flow even in rodents with millisecond temporal resolution, should allow it to become a useful and valuable tool to understand the effects of IUGR on the brain in clinical and in preclinical settings. We have applied fUS to study the brains of rats in a paradigm of protein restriction-induced IUGR and found that it identified connectivity deficits (59), and was able to validate improvements in connectivity associated with neuroprotection. For further comprehensive information on the applications of fUS, please see the recent review by Deffieux et al. (247).

Genomic Analysis as a Means to Diagnose, Stratify Risk and Understand Disease Mechanisms

Genomic screening offers the promise of early and specific diagnosis, risk stratification and personalized therapeutic deployment. There are inherent ethical issues with genetic screening that are in no way limited to the field of IUGR. These include incidental findings of unknown significance and the more complex issue of the rights of the child, and specifically the child's right "not to know." Violations of the child's rights may relate to the discovery of gene variants that have no bearing on fetal development or childhood health, but which alter adult disease risk. Although generally these problems are overcome with targeted analysis, variants with impact at multiple stages of life make this problem harder to manage. For a lively discussion on bioethics related to prenatal genome sequencing we refer the reader to these references (248–250).

Despite these concerns, non-invasive prenatal screening (NIPS) for fetal aneuploidies using cell-free DNA has been widely adopted in clinical practice due to its improved accuracy compared to traditional screening approaches (251). NIPS approaches can include whole genome sequencing techniques with high sensitivity and specificity even with very low sample input (252). The benefits of whole-genome sequencing include the identification of micro-deletions that would be missed by karyotyping. These micro-deletions are relevant to human growth and have identified some genetic causes of IUGR (253). It might not be necessary to sequence the genome to identify disease risk but to examine single nucleotide polymorphisms (SNPs). SNP analysis is increasingly accessible with sequencing, and whole-genome SNP arrays can be processed within 3 days (254). This short time frame means that the data can affect prenatal diagnostic decisions, and in the future, may guide treatment decisions. An approach to risk stratification has been tested in the related field of preterm birth, identifying an unbiased association of SNPs with white matter imaging phenotype. This approach has successfully identified several SNPs that associate with a clinical white matter phenotype in preterm infants—namely, specific SNPs within the genes for *FADS2* (Fatty Acid Desaturase 2) (255), *PPARG* (Peroxisome proliferator-activated receptor gamma) (256), and *DLG4* (discs large homolog 4, the gene Post-Synaptic Protein-95) (257). Although these SNPs provide a biomarker for infant outcome, they are also under further investigation to understand the mechanistic link between these genes variants and brain development.

What is lacking in the field of IUGR to date is the application of these genomics approaches in massive populations to identify variants denoting risk of obstetric and (neuro)developmental outcomes. In the fields of Alzheimer's disease and cancer, it has taken the study of many thousands of patients (and their tumors) to identify risk phenotypes that are now used to drive drug discovery efforts and to identify targets for personalized drug therapy, and these approaches have been effective in public health (258). Altogether, dramatic improvements in technology open avenues for understanding three important aspects of IUGR—diagnosis, risk stratification, and mechanisms of disease. The

logistical and monetary support to bring together a vast number of patients across multiple centers, and the bioinformatics needed to understand these processes, are now within reach if we apply the lessons from genetics studies of adult diseases.

CONCLUSION

IUGR remains a complex health care issue across the globe. Concerted research and clinical effort have raised the profile of this complex problem and improved our knowledge of the neurodevelopmental sequelae of IUGR. What we cannot yet offer in the field of IUGR is an animal model that recapitulates the underlying pathophysiology of IUGR, primarily because we do not yet know the etiology (probably, more than one etiology) underlying the various sub-groups of IUGR. Valuable post-mortem studies, imaging studies using newly optimized *in utero* fetal and placental imaging, and pooling our research data across models to find common elements, will be key to overcoming this hurdle. Optimized fetal and placental imaging will also be important future methods of early diagnosis and risk stratification. These will enable us to treat the IUGR infant *in utero* with the goal of preventing brain injury rather than attempting postnatal repair or regeneration. We do not yet understand the role of neuroinflammation in the human IUGR infant, and as such cannot determine if the multitude of immunomodulatory drugs being fast-tracked in fields such as multiple sclerosis and Alzheimer's disease apply to the IUGR infant. The role of neuroinflammation can be studied by bringing together multiple models capturing many facets of IUGR-related neurodevelopmental injury, and more post-mortem studies. We are also limited in our ability to target drugs to either the placenta or the fetus, but these issues will be overcome by advances in materials bioengineering, and proof-of-concept drugs and tools entering the fields of adult medicine. Given the vast numbers of infants born IUGR due to preventable causes such as malnutrition, malaria, HIV infection, and even psychosocial stress such as domestic abuse, we already have a mandate to reduce its impact across the world. Initiatives such as the Millennium Development Goals are making significant improvements in these areas but require our further support.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

The funders played no role in the decision to publish or the content of the publication. (Cerebral Palsy Alliance; National Health and Medical Council of Australia; Australian Research Council; ANR—French Research Agency).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 09 April 2019
doi: 10.3389/fendo.2019.00215

Cardiovascular Programming During and After Diabetic Pregnancy: Role of Placental Dysfunction and IUGR

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *07 November 2018*
Accepted: *18 March 2019*
Published: *09 April 2019*

Citation:
Langmia IM, Kräker K, Weiss SE, Haase N, Schütte T, Herse F and Dechend R (2019) Cardiovascular Programming During and After Diabetic Pregnancy: Role of Placental Dysfunction and IUGR. Front. Endocrinol. 10:215. doi: 10.3389/fendo.2019.00215

Intrauterine growth restriction (IUGR) is a condition whereby a fetus is unable to achieve its genetically determined potential size. IUGR is a global health challenge due to high mortality and morbidity amongst affected neonates. It is a multifactorial condition caused by maternal, fetal, placental, and genetic confounders. Babies born of diabetic pregnancies are usually large for gestational age but under certain conditions whereby prolonged uncontrolled hyperglycemia leads to placental dysfunction, the outcome of the pregnancy is an intrauterine growth restricted fetus with clinical features of malnutrition. Placental dysfunction leads to undernutrition and hypoxia, which triggers gene modification in the developing fetus due to fetal adaptation to adverse *utero* environmental conditions. Thus, *in utero* gene modification results in future cardiovascular programming in postnatal and adult life. Ongoing research aims to broaden our understanding of the molecular mechanisms and pathological pathways involved in fetal programming due to IUGR. There is a need for the development of effective preventive and therapeutic strategies for the management of growth-restricted infants. Information on the mechanisms involved with *in utero* epigenetic modification leading to development of cardiovascular disease in adult life will increase our understanding and allow the identification of susceptible individuals as well as the design of targeted prevention strategies. This article aims to systematically review the latest molecular mechanisms involved in the pathogenesis of IUGR in cardiovascular programming. Animal models of IUGR that used nutrient restriction and hypoxia to mimic the clinical conditions in humans of reduced flow of nutrients and oxygen to the fetus will be discussed in terms of cardiac remodeling and epigenetic programming of cardiovascular disease. Experimental evidence of long-term fetal programming due to IUGR will also be included.

Keywords: diabetes, placental dysfunction, gene modification, intra uterine growth restriction, cardiovascular programming

FETAL CARDIOVASCULAR PROGRAMMING IN CONDITIONS OF PLACENTAL DYSFUNCTION AND IUGR

Cardiovascular disease (CVD) is the main cause of mortality and morbidity in the twenty-first century (1–3). According to the World Health Organization (WHO), coronary heart disease and stroke accounted for ∼15.2 million deaths in 2016 globally. Previously, CVD was assumed to be caused by a series of events that occur after birth, such as a person's lifestyle, age, and other disease conditions. In other words, postnatal gene-environment interactions are important factors promoting CVD, but recently we began to understand that *in utero* environmental changes that occur in the first few months of prenatal development epigenetically program the fetus for development of CVD in adulthood (4–6). Therefore, apart from postnatal gene-environment interaction, information on *in utero* gene-environment interactions is needed to improve our understanding of CVD.

The placenta performs many functions ranging from growth of the fetus, prevention of fetal rejection by maternal immune system, as well as the transport/exchange of gases, nutrients, and waste products between mother and fetus (7). In addition, the placenta is involved in metabolism and production of many hormones that play vital roles in the maintenance of pregnancy (7). In a normal pregnancy the placental weight and birth weight are highly correlated (8). In adverse situations where the maternal-fetal circulation is altered due to disease conditions such as diabetes, the development of the placenta also changes (9). The determining factor in placental dysfunction is the extent of exposure to hyperglycemia during fetal and placental development (10). Depending on the degree of hyperglycemia, the growth of the placenta and the fetus can be severely affected (10). Long-term uncontrolled hyperglycemia can lead to placental vascular dysfunction in women suffering from diabetes (10). Endothelial dysfunction was observed in hyperglycemic human umbilical vein cells (HUVEC) compared to control cells (11). According to studies by Starikov et al. pre-gestational diabetes led to placentas that were small for gestational age in 20% of pregnancies and placentas that were large for gestational age in 30% of cases (9). In conditions where maternal hyperglycemia is not controlled, it results in histological changes of the placental tissues (10, 12). Placentas from mothers with diabetes display different histological features such as immature villous, increased number of fetal capillaries, and fibrinoid necrosis of the placental villi (7). Prolonged maternal insults such as hyperglycemia, dyslipidemia, and hyperinsulinemia may exceed the placental capacity to adapt and respond leading to placental dysfunction and adverse fetal outcome such as in IUGR (13, 14). Placental dysfunction is the most common cause of asymmetrical intrauterine growth restriction (15–17). Uteroplacental malperfusion, decidual vasculopathy, placental infarct, and maternal vasculopathy were observed in women with diabetes (12) and are associated with reduced fetal growth and adverse outcome (17, 18). Abnormal placental vascular development decreases normal placental blood supply, leading to reduced oxygen (hypoxia) and nutrient delivery (undernutrition) to the fetus and a subsequent IUGR fetus (16, 17). As a result of reduced oxygen and nutrition, the fetus redistributes its cardiac output to increase oxygen and nutrient supply to the brain, which is referred to as brain sparing (19). This prenatal cerebral circulatory adaptation has been associated with long-term behavioral problems in schoolchildren who were growth-restricted at birth. These difficulties include problems in memory, cognition, motor skills, and neuropsychological malfunction (20). IUGR offspring experience the highest rate of coronary heart disease, myocardial dysfunction, type 2 diabetes, hypertension, and stroke as adults (21, 22). The underlying molecular mechanisms leading to fetal susceptibility to adult disease are still under investigation. Interestingly, we now know that clinical symptoms of cardiovascular disease may not appear until adult life (4). In 1989, David Barker demonstrated this aspect amongst individuals with low birth weight, showing a direct association between low birth weight and CVD in adulthood. This study demonstrated that the incidence of ischemic heart disease and death were three times higher among men with low birth weight compared to men with high birth weight (5). Epidemiological investigations of adults born at the time of the Dutch famine between 1944 and 1945 revealed an association between maternal starvation and a low infant birth weight with a high incidence of hypertension and coronary heart disease in these adults (23). Furthermore, Painter et al. reported the incidence of early onset coronary heart disease among persons conceived during the Dutch famine (24). In that regard, Barker's findings led to the concept of fetal adaptation to prenatal environmental changes and vulnerability to chronic diseases in adult life, a concept known as "fetal programming." In 2003, the concept of "Developmental origins of health and disease (DOHaD)" was published (25). DOHaD describes the scenario whereby *in utero* maternal insults result in structural and functional changes in fetal organs extending in postnatal life and increasing susceptibility of chronic disease in adulthood. Fetal programming of other organs such as the brain (19), lungs (26), and hypothalamic-pituitary-adrenal (HPA) axis have been greatly studied, however the heart is also very important and few studies have investigated fetal programming of the heart as summarized below (27).

FETAL PROGRAMMING OF THE HEART IN IUGR

The heart is the first organ to develop and function during embryogenesis (28). Fetal organs that develop early are more vulnerable to changes of the *in utero* environment than organs that develop later (29). There is a direct correlation between the amount of oxygen and nutrient supply to the fetus and cardiomyocytes development and function (29). Chronic hyperglycemia causes placental vascular dysfunction, which leads to reduced nutrient and oxygen supply to the fetus resulting in IUGR (10). Neonates exposed to IUGR present significant changes in cardiac morphology and subclinical myocardial dysfunction at birth (29). As such, in placental dysfunction due to maternal diabetes involving undernutrition and hypoxia, the developing myocardium undergoes structural and functional

changes referred to as cardiac remodeling (30). In 1982, Hochman and Bulkley were the first to use the term "remodeling" to describe the substitution of infarcted tissue with scar tissue in a model of myocardial infarction (31). Later, in the year 2000, an international forum defined cardiac remodeling as molecular, cellular, and interstitial changes that manifest clinically as changes in size, shape, and function of the heart resulting from cardiac load and injury (30). Therefore, the clinical phenotype is a consequence of genomic changes that occur due to *in utero* gene environment interaction. Few clinical studies and experimental animal models have investigated the impact of IUGR on cardiac remodeling as discussed below.

IMPACT OF PLACENTAL DYSFUNCTION ON CARDIAC STRUCTURE AND FUNCTION

Epidemiological studies have shown changes in the structure and function of the heart in IUGR infants and neonates (29, 32). Clinical investigations have reported that postnatal catch-up growth in IUGR infants can result in abnormal cardiac function (33–35). Cardiovascular evaluation using echocardiographic assessment of IUGR fetuses revealed cardiac hypertrophy characterized by globular cardiac shape, higher intraventricular septum thickness, and increased pressure overload leading to both systolic and diastolic dysfunction (36–38). Primary cardiac and vascular changes observed at birth in IUGR fetus can persist from a few months up to a few years of age (35, 37).

Recently, clinical investigations have shown evidence of cardiac remodeling in IUGR offspring (32). For example, high systolic blood pressure and smaller aortic diameter (ascending aorta; IUGR:24.4 $\pm$ 1.5 mm, Control: 26.3 $\pm$ 2.2 mm, $P < 0.05$) were observed 20 years later in young adults (age 22–25 years) who were born growth restricted due to placental dysfunction (32). Compared to healthy controls, the diameter of the ascending aorta was smaller in IUGR subjects and a high aortic pressure augmentation index was observed (32). Electrocardiography (ECG) measurement revealed electrical remodeling in preadolescence who were born with IUGR (39). These studies evaluated signs of ventricular electrical remodeling in subjects with IUGR compared to controls by assessing changes in ventricular depolarization and repolarization using the QRS complex and *T*-wave, respectively (39). The classical frontal QRS-T angle was significantly narrower in IUGR preadolescents compared to control subjects (39). They also reported wider angles between the depolarization dominant vector and the frontal XY body plane in preterm-IUGR subjects, resulting in significantly wider angles between depolarization and repolarization vectors (39). The studies concluded that ventricular electrical remodeling observed in the pre-adolescent subjects might predispose to cardiovascular disease in later life (39). Electrical remodeling is induced by functional (altered electrical activation) and structural stressors (such as in hypertrophy and heart failure). These electrophysiological changes generate a substrate that is vulnerable to malignant ventricular arrhythmias. Cardiac remodeling and dysfunction increases with greater severity of IUGR (33, 40). The above-mentioned studies show evidence of cardiac remodeling in neonates, pre-adolescents, and young adults who were born with IUGR (41–44). These findings are supported by animal models; IUGR programming resulted in myocardial remodeling, reduced systolic and diastolic function and premature aging of the heart in growth restricted baboons (45). These structural and functional changes of the heart can be associated to changes in fetal cardiac gene expression that have occurred because of fetal adaptations to intrauterine environmental changes (46). These *in utero* changes, which result in reduced nutrition and oxygen, are due to placental dysfunction caused by maternal insults such as maternal vasculopathy in a diabetic pregnancy (46).

DIFFERENTIAL EXPRESSION OF CARDIAC GENES LEADING TO DYSFUNCTIONAL HEART IN IUGR

Pre-gestational and gestational diabetes have been associated with maternal vasculopathy which can be caused by long-term poor glycemic control (12). Fetal growth in diabetic pregnancy might be affected in two different ways; maternal hyperglycemia stimulates fetal overgrowth and maternal vasculopathy may be associated with placental dysfunction leading to a reduced nutrient and oxygen supply and subsequent IUGR (12). The majority of animal models use either maternal undernutrition and/or hypoxia to mimic IUGR (6, 47, 48). Hypoxia, undernutrition, or both interventions induce cardiac remodeling in adult rats (47). IUGR models have linked cardiac remodeling with changes in fetal gene expression. Several genes that play key roles in cardiac development have been studied in detail. One prominent example is the hypoxia-inducible factor 1 (HIF-1), which is required for normal growth of the myocardium and coronary blood vessels in conditions of low oxygen (49, 50). High levels of HIF-1 were reported in fetal rodent hearts exposed to hypoxia (49, 50). Abnormal cardiogenesis was seen in HIF-1 alpha-deficient mice (51). Therefore, elevated expression of *HIF1* and its downstream genes is essential for fetal adaptation and proper cardiac development in conditions of placental dysfunction in which oxygen supply to the fetus is relatively low (51–55). In a guinea pig model of maternal nutrient restriction, hearts of growth restricted offspring revealed increased expression of Poly [ADP-ribose] polymerase 1 (*PARP1)* gene and a reduction in cardiomyocyte number as well as hypertrophy (56). Another interesting class of genes associated with cardiac remodeling are the cardioprotective genes such as heat shock protein 70 (*HSP70*) and protein kinase C epsilon (*PKCε*) (57). Male rats exposed to hypoxia had increased expression of HSP70 and ischemic injury (48, 58–60). In addition, hypoxia in rat hearts resulted in a significant increase in *PKCε* expression and increased susceptibility to ischemia and perfusion injury in a sex-dependent manner (61). Furthermore, the mammalian target of the rapamycin complex 1 (mTORC1) pathway has also been associated with cardiac remodeling in IUGR mice, and prenatal *mTORC1* inhibition caused a reduction in cardiomyocyte number in adult hearts compared to controls

(62). In a rabbit model, IUGR was associated with cardiac mitochondrial Complex II dysfunction and an increase in Sirtuin 3 expression (63). In a rat model, maternal diabetes was associated with increased expression of forkhead box protein O1 (*FOXO1)* and its target genes in the heart of the offspring (64). Under conditions of undernutrition and hypoxia, changes that occur to enable the growing fetus to adapt and survive the inadequate substrate supply results in programming of fetal cardiac genes (4, 6, 65–67).

ROLE OF EPIGENETICS IN CARDIAC PROGRAMMING

Researchers have been investigating the mechanisms by which IUGR caused changes in fetal cardiac genes. Epigenetic regulation is one principle underlying molecular mechanism that causes differential gene expression in IUGR fetuses compared to normally grown fetuses. Epigenetic activities can alter gene expression throughout an individual's lifetime; thus, the function of the corresponding protein products as well as the organs involved are affected. Gene modifications occur in response to environmental changes, and genes are switched on and off via epigenetic mechanisms. Epigenetic alterations could be altered by lifestyle (for example overnutrition or undernutrition) or possibly by using treatments strategies postnatally (68). Hence, it is proposed that epigenetic mechanisms are important for understanding pathophysiology as well as potential targets for diagnosis and treatment of cardiovascular diseases (69, 70). Epigenetic regulation occurs by DNA methylation, histone modification, or regulation of genes by non-coding RNAs (ncRNAs) such as small microRNA and long non-coding RNA (71). So far, DNA methylation and histone acetylation are the most studied epigenetic mechanisms and clinical evidence of epigenetic programming is reported in adult patients with various cardiovascular diseases as reviewed by Muka et al. (72). Other studies have investigated the posttranscriptional regulatory role of non-coding RNA in relation to cardiovascular disease susceptibility in adult cardiac patients (73–75). We will focus on DNA methylation in IUGR leading to cardiovascular disease.

DNA METHYLATION MECHANISMS IN IUGR LEADING TO DEVELOPMENT OF CARDIOVASCULAR DISEASE

DNA methylation is the covalent addition of methyl groups to the C5 position of cytosine in dinucleotide CpG islands (76). CpG islands are regions with a high frequency of CpG sites where a cytosine nucleotide is followed by a guanine nucleotide. Methylation of CpG sites is catalyzed by DNA methyl transferases (DNMTs), such as DNMT1, DNMT3a, and DNMT3b. Very few studies have examined DNA methylation mechanisms in IUGR leading to cardiovascular remodeling or cardiac dysfunction. In a UK cohort of 144 children, investigators used umbilical cord blood to examine the relationship between prenatal antisense non-coding RNA in the INK4 locus (*ANRIL)* promoter DNA methylation and risk markers of coronary heart disease (76). Hypermethylation at CpG5 of the *ANRIL* promoter was associated with increased childhood pulse wave velocity, indicating increased arterial stiffness and a risk of coronary heart disease in these children at 9 years old (76). The *ANRIL* promoter is present on chromosome 2p21 which is considered a strong candidate for coronary heart disease in adult patients (77). Methylation of *ANRIL* and other genes on this chromosome might contribute to the prolonged cardiovascular programming in coronary disease patients. However, it is difficult to conclude the epigenetic contribution unless studies involving long-term follow-up of IUGR individuals are performed. Several studies have investigated epigenetic disturbances of nitric oxide synthase (NOS), which may predispose individuals to cardiovascular disease by modulating endothelial dysfunction (78–80). Human endothelial cells isolated from umbilical arteries and veins of IUGR fetuses were analyzed for a DNA methylation pattern in the promoter region of the endothelial nitric oxide synthase 3 (*eNOS3*) and arginine-2 (*ARG2*) genes. Compared to control cells, differential DNA methylation at the CpG-352 site of *NOS3* gene promoter of IUGR-endothelial cells was observed (80). Differential *eNOS* expression could be normalized by simply silencing the DNA methylation machinery (80). Hypoxia downregulates *eNOS* gene expression and activity, resulting in a reduced production of nitric oxide and endothelial dysfunction (78). Endothelial dysfunction is a very early stage in the development of atherosclerosis, which appears prior to the existence of atherosclerotic plaques or cardiovascular outcomes (81–84). This favors the concept that an epigenetic marker detectable as early as during prenatal and early postnatal developmental periods might be capable of identifying persons at risk of endothelial-related cardiovascular end organ damage. Furthermore, it has been shown that hypoxia-related IUGR changes the methylation pattern and expression of the *PKCε* gene in rat hearts, causing ischemic injuries in offspring (85). Hypoxia causes increased production of reactive oxygen species (ROS) which induces epigenetic repression of the PKCε gene leading to susceptibility of the heart to ischemic injury in the offspring (85). Hypermethylation patterns were observed within the promoter region of the *PKCε* gene in IUGR male rat hearts compared to controls (85). *PKCε* promoter hypermethylation was associated with a corresponding down regulation of *PKCε* gene expression in the heart of male rats but not in females. Activation of the *PKCε* gene in the heart restored hypoxia-induced cardiac vulnerability to ischemic injury in male rats (85). The transcription factor, early growth response factor (Egr-1), is involved in the regulation of *PKCe* promoter activity (86) since it binds to the *PKCε* promoter region, increasing its activity. Further studies showed that the absence of methylation in females was due to high levels of estrogen receptors in the female rat heart. Estrogen receptors bind to the regulatory gene Egr-1 and inhibit *PKCε* promoter methylation activity (86). Therefore, compared to males, the female hearts are protected against hypoxia-induced ischemic injury due to high levels of estrogen. These data help to explain the differences in the prevalence of CVD between men and women and gives a molecular mechanism for the role of sex hormone

(87). Postmenopausal women seem to lose this hormonally-induced cardioprotective ability and as such, hormone therapy is suggested as one of the remedies for prevention of cardiovascular disease in this group of women (88). However, large studies do not favor hormone replacement therapy for prevention of cardiovascular disease since it also has side effects such as increased risk for breast cancer and pulmonary embolism (88). For IUGR epigenetically programmed hearts, the best preventive strategy would be the use of epigenetic therapies (drugs) that aim to reverse fetal heart programming in early postnatal life (89). Postnatal drugs are capable of altering the epigenetic modifications that occurred during prenatal life as a result of fetal adaptations to changes in the intrauterine environment (89). Animal models and human studies of IUGR revealed epigenetic changes in 10 genes which result in a predisposition to type 2 diabetes as reviewed in detail by Liguori et al. (90). These epigenetic mechanisms may further contribute to the high prevalence of cardiovascular disease in type 2 diabetes patients (90). Another gene involved in growth and development of the fetal heart is insulin-like growth factor *(IGF*). Upregulation of cardiac IGF receptor is associated with an increase in ventricular mass in a sheep model of nutrient restriction (91). Moreover, epigenetic mechanisms in the renin angiotensin system are linked to cardiovascular programming. Animal models revealed epigenetic modification of the renin-angiotensin system in fetal programming of hypertension (92, 93). Significantly lower renal expression of angiotensin II type 2 receptor, impairment of renal development and elevation of blood pressure were observed (93). Maternal insults during pregnancy induce placental dysfunction, leading to IUGR (**Figure 1**) (10). So far, it is well established that IUGR leads to cardiac remodeling via epigenetic modulation of cardiac genes which play important roles in cardiac development and function. However, many epigenetic pathways are still to be elucidated.

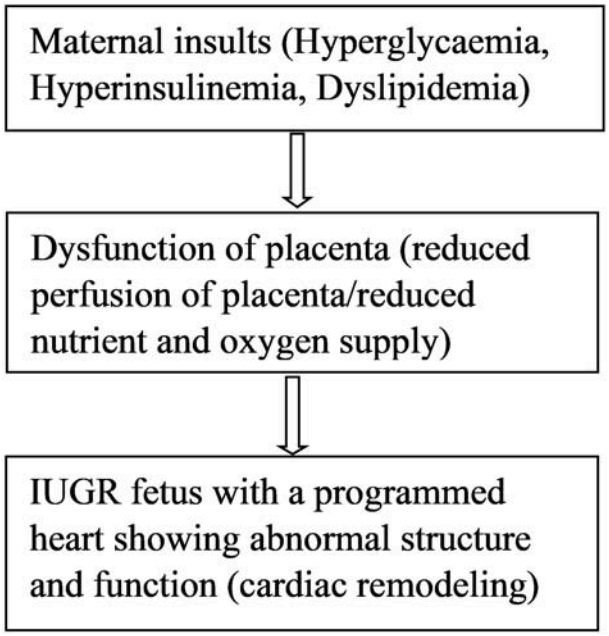

FIGURE 1 | Maternal insults result in placental dysfunction, IUGR, and cardiac remodeling.

DISCUSSION AND FUTURE PERSPECTIVE

Placental dysfunction due to maternal insults can result in IUGR, which result in an altered fetal epigenome leading to cardiac remodeling and subclinical symptoms of cardiovascular phenotypes in offspring. It is well-proven that IUGR leads to cardiac remodeling, likewise evidence linking altered genome expression and cardiovascular disease in IUGR has been published (85, 86, 91, 94). However, research involving epigenetic mechanisms of cardiovascular programming in growth restricted fetuses has just commenced. To date, most research has focused on fetal programming leading to CVD-related risk factors such as a predisposition to metabolic syndrome (95–97). Although such studies improve our understanding of how to prevent cardiovascular disease in carriers of cardiovascular risk factors, it is important to fully understand the epigenetic mechanisms acting directly on cardiovascular structure and function in IUGR offspring. It seems IUGR results in DNA methylation in a tissue-specific manner; thus, understanding the epigenetic mechanisms in various tissues that have direct impact on the cardiac system will be beneficial (98). The greatest task is to uncover the specific pathways of prenatal methylation of gene expression, as it is vital to the development and function of heart tissues, and to be able to link these pathways to specific cardiac diseases. Even though one can find DNA methylation in interesting CpG island in clinical samples obtained from affected IUGR offspring, it is also difficult to conclude a causal link between these epigenetic modifications and the various heart diseases. However, animal models may help to improve our understanding. Another challenge is linking affected pathways based on the severity of the IUGR since the extent of cardiac remodeling is based on the severity of IUGR (40). Epidemiological time point experiments have been performed to widen our understanding on the role of IUGR in fetal vulnerability to cardiovascular disease (33–35); however, there is need for longitudinal studies involving long-term follow-up of affected IUGR offspring. The use of epigenetic therapies in treatment of CVD is promising but it is still at its early experimental stage. In other diseases such as cancer, systemic lupus erythematosus, acute myeloid leukemia, and Alzheimer's disease, many epigenetic-related therapeutic agents are being tested for their possible use in clinical practice, but many are still awaiting approval to begin clinical trials or to receive approval by the FDA (99–102). In terms of DNA methylation mechanisms, therapeutic agents such as inhibitors of DNA methyl transferase (DNMT), hormonal therapy, and certain dietary compounds have been suggested for treatment of cardiovascular disease (89). Early prediction of cardiovascular disease might reduce risk as well as improve follow-up and treatment of susceptible patients. Effective biomarkers of risk are needed for development of

CVD prevention strategies (103). Epigenetic biomarkers could be useful for early prediction of high-risk individuals (103).

Growth restricted newborns are at high risk of respiratory distress syndrome, retinopathy of prematurity, and long-term complications such as metabolic syndrome and cardiovascular disease (104, 105). Therefore, it is vital to identify and follow those who are likely to be affected. Studies also show that an inheritable altered fetal cardiac genome can be transferred from one generation to another, which is referred to as transgenerational programing of the cardiac system (106). "Healthy aging starts with a healthy pregnancy," summarizes very beautifully this important concept and was first introduced in an editorial of Scioscia (107). A timely diagnosis of IUGR pregnancies and application of various therapeutic measures to treat and monitor affected neonates will help to reduce cardiac problems in future generations.

AUTHOR CONTRIBUTIONS

RD and IL conceived the presented idea. IL wrote the first draft of the manuscript. KK, SW, NH, TS, FH, and RD contributed to the manuscript revision, read and approved the submitted version.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 18 April 2019
doi: 10.3389/fendo.2019.00238

More Maternal Vascular Malperfusion and Chorioamnionitis in Placentas After Expectant Management vs. Immediate Delivery in Fetal Growth Restriction at (Near) Term: A Further Analysis of the DIGITAT Trial

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Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: 23 October 2018
Accepted: 26 March 2019
Published: 18 April 2019

Citation:
Feenstra ME, Schoots MH, Plösch T, Prins JR, Scherjon SA, Timmer A, van Goor H and Gordijn SJ (2019) More Maternal Vascular Malperfusion and Chorioamnionitis in Placentas After Expectant Management vs. Immediate Delivery in Fetal Growth Restriction at (Near) Term: A Further Analysis of the DIGITAT Trial. Front. Endocrinol. 10:238. doi: 10.3389/fendo.2019.00238

Objective: Management of late fetal growth restriction (FGR) is limited to adequate fetal monitoring and optimal timing of delivery. The *Disproportionate Intrauterine Growth Intervention Trial At Term* (DIGITAT) trial compared induction of labor with expectant management in pregnancies at (near) term complicated by suspected FGR. Findings of the DIGITAT trial were that expectant monitoring prolonged pregnancy for 10 days and increased birth weight with only 130 grams. This resulted in more infants born below the 2.3rd percentile compared to induction of labor, respectively, 12.5% in induction of labor and 30.6% in expectant monitoring group. The main placental lesions associated with FGR are maternal vascular malperfusion, fetal vascular malperfusion, and villitis of unknown etiology. We investigated whether placentas of pregnancies complicated with FGR in the expectant monitoring group reveal more and more severe pathology due to pregnancy prolongation.

Material and methods: The DIGITAT trial was a multicenter, randomized controlled trial with suspected FGR beyond 36 + 0 weeks. We now analyzed all available cases ($n = 191$) for placental pathology. The macroscopic details were collected and histological slides were recorded and classified by a single perinatal pathologist, blinded for pregnancy details and outcome. The different placental lesions were scored based on the latest international criteria for placental lesions as defined in the Amsterdam Placental Workshop Group Consensus Statement.

Results: The presence of maternal vascular malperfusion and chorioamnionitis were higher in the expectant management group ($p < 0.05$ and $p < 0.01$, respectively). No differences in placental weight and maturation of the placenta between the induction of labor and the expectant management group were seen. Fetal vascular malperfusion, villitis of unknown etiology and nucleated red blood cell count did not differ between the groups.

Conclusion: Expectant management of late FGR is associated with increased maternal vascular malperfusion and chorioamnionitis. This may have implications for fetal and neonatal outcome, such as programming in the developing child influencing health outcomes later in life.

Keywords: fetal growth restriction, placental pathology, DIGITAT trial, maternal vascular malperfusion, chorioamnionitis

INTRODUCTION

Fetal growth restriction (FGR) is a condition in pregnancy in which the fetus fails to reach its growth potential. FGR affects up to 15% of all pregnancies (1, 2) and is not only associated with mortality, but also with long term morbidity (3–5). Appropriate placental nutrient and oxygen supply is essential for normal fetal growth. The origin of placental insufficiency is multifactorial (6, 7). Several types of lesions can be found in placentas of pregnancies complicated by FGR (7–9). The main placental lesions found in FGR placentas are maternal vascular malperfusion, fetal vascular malperfusion, and villitis of unknown etiology (9). Elevated nucleated red blood cells are considered as an indication of fetal hypoxia (10, 11). Other rare findings in placentas of FGR complicated pregnancies include chronic histiocytic intervillositis and massive perivillous fibrinoid deposition (12, 13).

Until now, adequate fetal monitoring and optimal timing of delivery are the only possible interventions for FGR as other therapeutic options are lacking (14). Timing of delivery in case of FGR is balanced between (relative) prematurity on the one side and prolonged undernutrition/hypoxia on the other side. The DIGITAT study investigated whether timely delivery in (near) term FGR would result in better outcomes. The primary analysis (36 + 0 till 41 + 0 weeks of gestation) showed equivalence in composite neonatal outcome between expectant monitoring and induction of labor (15, 16). Sub analysis revealed that the optimal time to minimize neonatal consequences is delivery around 38 weeks of gestation, when benefits of a planned delivery outweigh consequences of prolongation of pregnancy, as this is associated with a longer exposure to a malnourished environment (17). The DIGITAT trial found that expectant monitoring resulted in a pregnancy prolongation of a further 10 days compared to those in the immediate induction group. This was associated with an increase of only 130 grams in the birth weight of those expectantly managed and a higher proportion of infants born below the 2.3 percentile (30.6% compared to 12.5% in the immediate induction of labor group).

In this study we investigate whether placentas of pregnancies complicated with FGR at (near) term show specific features due to prolongation of pregnancy. The aim of this study is to assess the structural impact on the placenta when pregnancy is prolonged in FGR at term.

MATERIALS AND METHODS

DIGITAT-Original Study Design

The *Disproportionate Intrauterine Growth Intervention Trial At Term* (DIGITAT) trial was a multicenter, randomized controlled trial of women pregnant with a singleton fetus with suspected FGR from 36 + 0 till 41 + 0 weeks of gestation onwards. Details of the DIGITAT trial protocol have been described elsewhere (15). In brief, consenting and eligible women were randomized to either expectant monitoring or induction of labor. The expectant monitoring group was monitored until the onset of spontaneous delivery or when for other reasons than suspected FGR an indication for delivery became apparent. In the induction of labor group delivery was induced within 48 h after randomization (17, 18).

Data Collection of Further Analysis (Current Study)

All participating hospitals in the DIGITAT trial were asked to evaluate whether the placentas of their trial participants had been examined by a local pathologist. If so, all available material such as pathology reports, histologic slides, and tissue blocks were collected and re-analyzed. The analyses included details of the macroscopic description of the placental disk, membranes and umbilical cord as described in the placental pathology report.

Furthermore, the histologic slides, routinely stained with Hematoxylin and Eosin (H&E) were revised, scored and classified to latest international criteria (9) by a single perinatal pathologist (MS), who was blinded for pregnancy details and outcome.

Placental Histology

The various placental lesions were evaluated based on the latest international criteria for placental lesions as described in the Amsterdam Placental Workshop Group Consensus Statement (9). Lesions evaluated were placental pathology consistent with maternal vascular malperfusion (MVM), fetal vascular malperfusion (FVM), chorioamnionitis (CA), villitis of unknown etiology (VUE), elevated nucleated red blood cell count (NRBC), chronic histiocytic intervillositis (CHI), and massive perivillous fibrin deposition (MPVFD). Also, if available, placental weight, placental weight percentile, birth weight placenta weight ratio (BWPW) (19), umbilical cord length and coiling index were included in the analyzes. The coiling index was calculated by dividing the number of whole coils by the umbilical cord length in centimeters. A normal coiling index is considered to be between 0.1 and 0.3. Due to the retrospective nature of this analysis unfortunately no other placental characteristic data (like the extension of infarction) were available.

Statistical Analysis

SPSS 24.0 software for Windows (SPSS Inc. Chicago, IL) was used for the statistical analyses. Continued variables were evaluated with a two-tailed unpaired *T*-test and binary or categorical

variables were evaluated with a Fisher Exact Test. Statistical significance was assumed at $p < 0.05$.

RESULTS

A total of 191 cases were available for review of placental pathology, respectively, 97 (321 in the original DIGITAT trail) participants in the induction of labor group and 94 (329 in original DIGITAT trail) participants in the expectant monitoring group.

In contrast to the original DIGITAT study the baseline characteristics of the two groups were statistically different for parity, maternal smoking and BMI (**Table 1**). Similar to the original DIGITAT study gestational hypertension was significantly higher in the expectant monitoring group. In the DIGITAT trial pre-eclampsia was more prominent in the expectant monitoring group than in the induction of labor group, but in our cohort of this re-analysis no significant difference was seen in the presence of pre-eclampsia.

Most women in the current analysis met the DIGITAT study criteria of FGR by combinations of inclusion criteria, usually by a fetal abdominal circumference (AC) below the 10th percentile (respectively $n = 74$ in induction of labor and $n = 77$ in expectant monitoring) and/or an estimated fetal weight (EFW) below the 10th percentile (respectively $n = 92$ in the induction of labor and $n = 72$ in the expectant monitoring). Furthermore, a decline in AC growth was also a criterium for study inclusion (not as a single criterium, but in combination with either AC or EFW below p10) and was differently observed between the groups, with a higher presence in the expectant monitoring group (induction of labor was $n = 14$ and expectant monitoring $n = 24$; $p < 0.05$) (**Table 1**).

In the expectant monitoring group in 51 of the 94 cases labor was induced due to either maternal indication (8), fetal indication (20), combined maternal and fetal indication (4), or rupture of membranes (2) (**Table 1**).

In the current analysis, women in the expectant monitoring group had a longer gestational age (8 days), compared to the induction of labor group ($p < 0.01$). This is slightly less prolongation than in the original DIGITAT study in which pregnancy was prolonged by 10 days. Birth weight in the study population of this sub analysis was not significantly different between the groups. However, there was a difference of 60 grams between the expectant management and induction of labor, which means a weight gain of 60 grams in the achieved 8 days of pregnancy prolongation. This is also slightly less than in the original DIGITAT study (with 130 grams weight gain in 10 days) (**Table 1**). The average neonatal stay in hospital was approximately 8 days in both groups. A few cases needed admission to the neonatal intensive care unit, significantly more in the expectant monitoring group ($p < 0.05$). The length of stay in the neonatal intensive care unit was approximately 6 days for both groups. There were no neonatal deaths reported in both groups.

Placental Characteristics

In **Table 2** placental characteristics are described of both the induction of labor group and the expectant monitoring group. No differences in placental weight, umbilical cord length, coiling index and BWPW ratio were found between the two groups.

Placental Histology

The findings of placental histology in both groups are described in **Table 3**. Maturation of the placenta was similar in both groups. Maternal vascular malperfusion (MVM) was significantly higher in the expectant monitoring group compared to the induction of labor group ($p < 0.05$). Fetal vascular malperfusion (FVM) was similar in both groups. In placentas from the expectant monitoring group we found a significantly higher incidence of chorioamnionitis (CA) ($p < 0.01$). The presence of villitis of unknown etiology (VUE) and the nucleated red blood cell count (NRBC) was similar in both groups. Rare placental findings like chronic histiocytic intervillositis (CHI) and massive perivillous fibrinoid deposition (MPFD) were seen in a few cases ($n = 2$ for CHI in the induction group and 0 in the expectant monitoring group; $n = 4$ and $n = 1$ for MPFD in the induction group and expectant monitoring group, respectively).

DISCUSSION

In this sub analysis of the DIGITAT study we found that maternal vascular malperfusion (MVM) occurred significantly more often in the expectant monitoring group in comparison to the induction of labor group ($p < 0.05$) in (near) term FGR. Moreover, participants in the expectant monitoring group had a significantly higher incidence of chorioamnionitis (CA) ($p < 0.01$). The DIGITAT trial previously showed no differences in composite neonatal outcome between expectant monitoring and induction of labor, however, it was seen that the average weight gain was only 130 grams in 10 days delivery delay, which resulted in more babies born with a birth weight below the p2.3. The follow up study of the original DIGITAT trial at 2 years of age showed that babies born with a birth weight below p2.3 scored lower at the ages and stages questionnaire (ASQ) for developmental disorders.

MVM occurs early in pregnancy and is likely due to maladaptation of the spiral arteries. It develops over time when gestation prolongs (21, 22). By inadequate remodeling of spiral arteries a high resistance flow induces shear stress and damage to parenchyma, like infarctions, and differences in oxygen tension leading to oxidative stress and free radical damage (12, 22–25). The higher prevalence of MVM in the expectant monitoring group could be caused by a prolonged time of hypoxia caused by inadequate maternal vascularization. This could further lead to increase in cellular stress, e.g., oxidative stress and endoplasmic reticulum (ER) stress. This may ultimately lead to an increase in the occurrence of FGR and development of preeclampsia (23–26).

To support this theory of prolonged exposure to a pathologic environment, a significant higher prevalence of the development of gestational hypertension in the expectant monitoring group was seen. MVM has been described to be causal in gestational hypertension and preeclampsia (20, 27). This finding further strengthens the clinical experience that expectant monitoring or prolongation of pregnancy in FGR at (near) term is not without

TABLE 1 | Demographic and baseline characteristics of the selected randomized participants for this analysis of placental pathology in FGR at term.

Baseline characteristics	Induction of labor ($n = 97$)	Expectant monitoring ($n = 94$)	Sig.
Nulliparous (%)	48	37	$p = 0.013$
Maternal age (years)	28.07 ± 5.58	27.65 ± 5.41	$p = 0.500$
BMI at study entry (kg/m^2)	24.31 ± 6.09	23.18 ± 4.82	$p = 0.024$
Maternal smoking	58	39	$p = 0.010$
Gestational hypertension	7	13	$p = 0.035$
Pre-eclampsia	6	9	$p = 0.150$
INCLUSION CRITERIA			
Fetal abdominal circumference <10th percentile	76	82	$p = 0.211$
Estimated fetal weight <10th percentile	95	76	$p = 0.224$
Deceleration of fetal abdominal circumference curve	14	26	$p = 0.038$
MODE OF DELIVERY			
Induced labor	95	51	$p = 0.001$
Vaginal delivery	43	39	$p = 0.100$
Vaginal instrumental	33	34	$p = 0.352$
Cesarean section	24	27	$p = 0.248$
NEONATAL CHARACTERISTICS			
Gestational age at birth (days)	264 ± 7.71	272 ± 9.27	$p = 0.010$
Birth weight (grams)	2242.23 ± 285	2303.10 ± 365	$p = 0.065$
Length of neonatal stay in hospital (days)	8.9 ± 7.00	9.1 ± 8.41	$p = 0.690$
Admission to the neonatal intensive care unit (NICU)	2	5	$p = 0.020$

Data is presented as proportion, unless stated otherwise in the table. Significance is presented as p-value, a $p < 0.05$ is assumed significant.

TABLE 2 | Comparison of placental characteristics between the induction of labor and expectant monitoring group in FGR at term.

Placental characteristics	Induction of labor ($n = 97$)	Expectant monitoring ($n = 94$)	Sig.
Placental weight (grams)	371.49 ± 15.50	361.40 ± 15.54	$p = 0.707$
Umbilical cord length (cm)	35.44 ± 1.92	35.36 ± 1.93	$p = 0.789$
Coiling index	0.35 ± 0.189	0.30 ± 0.216	$p = 0.483$
Birth Weight to Placental Weight ratio (BWPW)	6.29 ± 1.336	6.71 ± 1.787	$p = 0.284$

Data is presented as mean. Significance is presented as p-value, a $p < 0.05$ is assumed significant. No statistical differences were found in placental characteristics.

TABLE 3 | The comparison of placental pathology between induction of labor and expectant monitoring in FGR at term.

Placental histology	Induction of labor ($n = 97$)	Expectant monitoring ($n = 94$)	Sig.
Maturation matching gestational age (MAT)	89	82	$p = 0.701$
Maternal vascular malperfusion (MVM)	9	17	$p = 0.049$
Fetal vascular malperfusion (FVM)	10	16	$p = 0.069$
Chorioamnionitis (CA)	13	32	$p = 0.010$
Villitis of unknown etiology (VUE)	29	30	$p = 0.507$
Elevated nucleated Red Blood Cell Count (NRBC)	2	3	$p = 0.485$
Chronic histiocytic intervillositis (CHI)	2	0	$p = 0.257$
Massive perivillous fibrinoid deposition (MPFD)	4	1	$p = 0.194$

Data is presented as proportion in the table. Significance is presented as p-value, a $p < 0.05$ is considered significant.

risks especially for the mother (28), even though on short term no differences were seen in neonatal outcome. A study from Parra-Saavedra et al. implies in a group of late onset Small-for-Gestational Age (SGA) that placental under perfusion (PUP) can also lead to higher neonatal morbidity; in this study in 77/84 placentas maternal vascular supply was—as in our study-compromised (29). Furthermore, similar to the original study, in this sub-study a large group of women had labor induced although being allocated to the expectant monitoring group (51/94: 66%), indicating that the difference between the groups in MVM is in fact an underestimation of what would really happen when delivery was awaited.

The histologic diagnosis of chorioamnionitis (CA) is made when an inflammatory infiltrate is seen in the chorioamniotic membranes. At term CA is most frequently induced by inflammation and cellular stress, in the absence of a microorganism (30). During labor the uteroplacental blood flow is compromised leading to hypoxia of the placenta and its membranes. This leads to an increase in pro-inflammatory markers in the placenta. In fact, labor at term has been associated with infiltration of inflammatory cells in the placenta and higher plasma levels of pro-inflammatory chemokines and cytokines, such as Interleukin 6 (IL-6), Interleukin 8 (IL-8), and Tumor Necrosis Factor alpha (TNF-alpha) (30–32). Hypothetically, the inflammatory response in the chorioamniotic membranes and

cervix could be the result of an increase in pro-inflammatory cytokines caused by cellular stress, e.g., oxidative stress and ER stress (23, 24, 33, 34).

Furthermore, even though in the expectant monitoring group labor was often induced, significantly more spontaneous deliveries took place in the expectant monitoring group. Recent studies showed a higher incidence of histological chorioamnionitis in spontaneous labor compared to, respectively, induced labor, or cesarean section (33, 35). This could be an additional explanation for the significantly higher incidence of chorioamnionitis in the expectant monitoring group.

In our analysis, we observed a higher prevalence of CA in the expectant monitoring group. As described above, this is conceivably explained by increase of pro-inflammatory cytokines due to the prolonged hypoxic environment.

The incidence of fetal vascular malperfusion (FVM) was not significantly different although a higher number of cases were seen in the expectant monitoring group in comparison with induction of labor.

Like MVM, FVM has been associated with fetal growth restriction (FGR) due to decreased availability of functional parenchyma (36). The etiology of FVM is however multifactorial and not specifically related to FGR. FVM has been associated with multiple factors as placental (for example umbilical cord pathology), maternal (for example preeclampsia), and fetal (for example cardiac malformations) etiologies (6, 37, 38). Also, intra-amniotic infection with a fetal response (vasculitis) and chronic villitis of unknown etiology (VUE) are known associations (39).

In our study, villitis of unknown etiology (VUE) occurred in approximately 30% of all cases, distributed equally between both randomized groups. In existing literature, a broad range of incidences of VUE is described, partly because the definition has not been uniform and also due to differences in sampling of placental parenchyma (40–43). With an equal distribution between our groups, we conclude that prolonged pregnancy in the expectant monitoring group is not associated with an increased prevalence of VUE.

At term, normally only a small number of circulating nucleated red blood cells (NRBCs) are seen in the fetal circulation, if any (11). In response to fetal hypoxia the NRBC count increases (10, 11). In our study, only a few cases showed elevated NRBCs, suggesting that fetal monitoring to prevent fetal hypoxia has been adequate in the two randomized groups as no differences were seen in number of cases with elevated NRBCs.

In addition to the placental lesions described above, we also investigated the presence of chronic histiocytic intervillositis (CHI), massive perivillous fibrinoid deposition (MPFD), and maternal floor infarction (MFI). These are rare placental lesions, associated with (severe) FGR, with high recurrence risks (12, 13, 44–47). In our analyses, we found 2 cases of CHI in the induction of labor group and none in the expectant monitoring group. Five cases of MPFD were encountered, four of which in the induction of labor group and one in the expectant monitoring group. No cases with maternal floor infarction (MFI) were found in the total study population. No significant differences were seen between the induction of labor group and the expectant monitoring group for CHI and MPFD.

We did not find any differences in placental weight or birth weight to placental weight ratio (BWPW ratio) (**Table 2**). It has been described that placental weight from fetuses with FGR is significantly lower than placentas from fetuses with a normal birth weight and that the BWPW ratio is higher, although in fact fetal weight per placental unit is high and the placenta performed well in that sense. A low BWPW ration (a relatively low fetal weight per placental unit) has also been described in relation to adverse outcome (19). Given the fact that pregnancy prolongation resulted in only little weight gain and placental weights were not different the BWPW ratio was, as expected lower (although not significant) in the induction group (8, 48, 49).

CONCLUSION

Placental pathology in FGR encompasses a broad group of diagnoses, not exclusive for FGR. Our study shows that expectant monitoring is associated with a higher incidence of maternal vascular malperfusion (MVM) and chorioamnionitis (CA), whilst fetal vascular malperfusion, severe acute fetal hypoxia and villitis of unknown etiology are similar in both groups. This study further strengthens the results from a previous sub-analysis that prolongation of gestation has no additional benefits and even increases the prevalence of MVM and CA in FGR; both placental findings are associated with an increase in children born with severe growth restriction and are possibly associated with unfavorable outcome at later ages.

Strengths and Limitations of the Study

The strengths of this study are a dedicated, uniform examination of the placentas, done by one perinatal pathologist unaware of clinical details, by the latest criteria, in a large number of placentas from pregnancies complicated with FGR. In this sub-study prolongation of pregnancy is 2 days less than the original DIGITAT study and the weight gain in that period is on average less per day (13 grams per day in the original study vs. 7.5 grams in this study). This combined with the fact that more inclusions showed a decline in AC growth than the original study and finally that more inductions in the expectant management group of this analysis (66%) occurred than in the original study (25.5%) indicates that there is probably an inclusion bias with the more severe cases included in the study. We however believe that this inclusion bias does not undermine the results of this study. In the induction group there may also be a similar trend in sending more placenta's of clinically severe cases to the pathologist than the clinically less severe cases. A limitation of this study is that only 29% cases of the original DIGITAT trial were included, due to the availability of placental histological samples, which were collected from all participating hospitals in the Netherlands. We believe it is of great importance to analyze the placenta histopathology for understanding the underlying pathophysiology, especially in cases of FGR in which a common mechanism is placental insufficiency. All in all, we found that expectant management of late FGR is associated with increased maternal vascular malperfusion and chorioamnionitis.

Further studies on the clinical implications of these findings are warranted.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of DIGITAT Guidelines, Leiden University Medical Centre. The protocol was approved by the METC LUMC P04.210.

AUTHOR CONTRIBUTIONS

MF, MS, JP, TP, SS, AT, HvG, and SG contributed to the design and implementation of the research, to the analysis of the results, and to the writing of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 22 May 2019
doi: 10.3389/fphys.2019.00560

Fetal Growth Restriction Alters Cerebellar Development in Fetal and Neonatal Sheep

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Fetal growth restriction (FGR) complicates 5–10% of pregnancies and is associated with increased risks of perinatal morbidity and mortality. The development of cerebellar neuropathology *in utero*, in response to chronic fetal hypoxia, and over the period of high risk for preterm birth, has not been previously studied. Therefore, the objective of this study was to examine the effects of FGR induced by placental insufficiency on cerebellar development at three timepoints in ovine fetal and neonatal development: (1) 115 days gestational age (d GA), (2) 124 d GA, and (3) 1-day-old postnatal age. We induced FGR *via* single umbilical artery ligation (SUAL) at ~105 d GA in fetal sheep, term is ~147 d GA. Animals were sacrificed at 115 d GA, 124 d GA, and 1-day-old postnatal age; fetuses and lambs were weighed and the cerebellum collected for histopathology. FGR lambs demonstrated neuropathology within the cerebellum after birth, with a significant, ~18% decrease in the number of granule cell bodies (NeuN+ immunoreactivity) within the internal granular layer (IGL) and an ~80% reduction in neuronal extension and branching (MAP+ immunoreactivity) within the molecular layer (ML). Oxidative stress (8-OHdG+ immunoreactivity) was significantly higher in FGR lambs within the ML and the white matter (WM) compared to control lambs. The structural integrity of neurons was already aberrant in the FGR cerebellum at 115 d GA, and by 124 d GA, inflammatory cells (Iba-1+ immunoreactivity) were significantly upregulated and the blood-brain barrier (BBB) was compromised (Pearls, albumin, and GFAP+ immunoreactivity). We confirm that cerebellar injuries develop antenatally in FGR, and therefore, interventions to prevent long-term motor and coordination deficits should be implemented either antenatally or perinatally, thereby targeting neuroinflammatory and oxidative stress pathways.

Keywords: cerebellum, preterm, term, fetal sheep, fetal growth restriction, brain injury, blood-brain barrier

OPEN ACCESS

Edited by:
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University of Nottingham,
United Kingdom

Reviewed by:
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University of Otago, New Zealand

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Physiology

Received: *19 November 2018*
Accepted: *24 April 2019*
Published: *22 May 2019*

Citation:
Yawno T, Sutherland AE, Pham Y, Castillo-Melendez M, Jenkin G and Miller SL (2019) Fetal Growth Restriction Alters Cerebellar Development in Fetal and Neonatal Sheep. Front. Physiol. 10:560. doi: 10.3389/fphys.2019.00560

INTRODUCTION

Fetal growth restriction (FGR) complicates 5–10% of pregnancies and is associated with increased risks of perinatal morbidity and mortality (Bernstein et al., 2000). FGR is linked to adverse perinatal outcomes, including stillbirth, prematurity, and need for neonatal intensive care; as well as long-term adverse outcomes including metabolic syndromes, hypertension, and neurological

deficits (Baschat, 2011; Mayer and Joseph, 2013; Albu et al., 2014; Drost et al., 2018). FGR is principally caused by placental insufficiency, which results in reduced utero-placental transfer of oxygen and nutrients to the developing fetus (Figueras and Gardosi, 2011). FGR can be induced experimentally to examine the neuropathology associated with FGR (Swanson and David, 2015). In sheep, restricted placental growth and function can be induced by surgical removal of the major endometrial caruncles from sheep before mating, causing a 31% reduction in body weight (Robinson et al., 1979; Harding et al., 1985). In rodents, uterine artery ligation models have been widely described in pregnant rats, guinea pigs, and mice (Basilious et al., 2015). Regardless of the experimental model, the fetus responds to chronic hypoxic challenge with a redistribution of cardiac output to favor the brain – termed brain sparing – but this does not ensure normal brain development (Miller et al., 2016). Chronic hypoxia results in widespread cerebral cellular and axonal lipid peroxidation, and white matter (WM) hypomyelination and axonal damage (Miller et al., 2014). FGR in lambs also induces cerebrovascular changes in the WM and causes disruption to the blood-brain barrier (BBB) (Castillo-Melendez et al., 2017). Developmental delays in motor and behavioral function are also present soon after birth in FGR lambs (Miller et al., 2014). These may be attributed to mal-development of the motor and coordination center within the brain, the cerebellum (Volpe, 2009). However, the *in utero* effects of placental insufficiency, chronic hypoxia, and FGR on cerebellar structure and development are not well understood.

The cerebellum is one of the first brain structures to differentiate, but one of the last to mature (Triulzi et al., 2005). In the last trimester of human gestation, and often coinciding with preterm birth in FGR offspring, cerebellar development undergoes a phase of rapid growth and interconnection with other cerebral structures (Volpe, 2009), which then continues into the first postnatal year (Abraham et al., 2001). Therefore, chronic hypoxia and/or preterm birth in the last pregnancy trimester may adversely impact cerebellar maturation and may also alter connectivity and subsequent development of other brain regions (Limperopoulos et al., 2010). The development of the cerebellum and cerebellar morphology has been described in fetal sheep (Rees et al., 1988, 1997). Purkinje cells are present by 100 days gestational age (d GA; term is 145–148 d GA) and branching of their dendritic tree is attained by 140 d GA. However, the presence of immature and mature dendritic neuronal markers indicates that considerable remodeling of Purkinje cell branching is still occurring in late gestation. Rat and guinea pig models of FGR show altered postnatal development of the cerebellum, including deficits in neuronal and glial formation (Mallard et al., 2000; So et al., 2013; McDougall et al., 2017). The ontogeny of cerebellar neuropathology *in utero*, in response to placental insufficiency and chronic fetal hypoxia, and over the period of high risk for preterm birth, has not been previously studied.

Therefore, the objective of this study was to examine the effects of FGR induced by placental insufficiency on cerebellar development. We induced late-onset FGR in fetal sheep *via* single umbilical artery ligation, which causes chronic fetal hypoxia and hypoglycemia but does not routinely cause preterm birth, as per late-onset FGR in the human (Baschat, 2014). We examined the cerebellum at three timepoints in ovine fetal and neonatal development; aim: (1) 115 d GA, (2) 124 d GA, and (3) 1-day-old postnatal age. Although the ontogeny of sheep cerebellar development is not well characterized with respect to human cerebellar development, we selected these timepoints to broadly correspond to human brain cerebrum and cerebellar development from 28 weeks through to infancy in the human (Rees et al., 1997; Back et al., 2012).

MATERIALS AND METHODS

Animal Surgery

The surgical and experimental procedures undertaken in this project were approved by Monash Medical Centre Animal Ethics Committee A (MMCA2010/03). Surgery was performed on 26 Border-Leicester-cross pregnant ewes carrying twins (Aims 1 and 2) or a single fetus (Aim 3; **Table 1**) to induce late-onset FGR *via* SUAL, as previously described (Miller et al., 2012; Alves de Alencar Rocha et al., 2017). Briefly, anesthetized ewes underwent surgery to instrument each fetus with a femoral artery catheter and an amniotic catheter for determination of blood gases and to administer antibiotics, respectively. The SUAL procedure was performed by ligating one of the umbilical arteries with two tight silk ligatures (FGR) and, in the sham procedure, the umbilical cord was exposed but not ligated. SUAL induces necrosis of approximately half of the placental cotyledons, thereby resulting in chronic placental insufficiency with reduced transfer of oxygen and glucose to the developing fetus (Miller et al., 2014). A maternal jugular vein catheter was inserted for antibiotic administration prior to recovery of the ewe.

Experimental Timeline

All animals were monitored daily from surgery until the day of postmortem. The ewe and fetuses were euthanased at three different ages to allow for comparisons of brain development and injury. At 115 and 124 d GA, animals were killed with maternal injection of pentobarbitone (Lethabarb; Virbac, Peakhurst, Australia) and the fetuses were removed, weighed, and brains were collected for analysis. In the third cohort, lambs were born naturally at term (~145 d GA) and at 24 h of age, lambs were euthanased with an intravenous injection of pentobarbitone; the lamb was weighed and brains were collected for analysis. The cerebellum of each animal was divided into right and left halves. The right half of the cerebellum was fixed by immersion in 4% buffered paraformaldehyde (PFA; ProSciTech, Thuringowa, QLD, Australia) for 3 days and transferred to 70% ethanol overnight, prior to embedding in paraffin. Subsequently, 10-μm coronal sections were cut for analyses.

Immunohistochemistry and Histological Stains

Sections were dewaxed and rehydrated through graded ethanol transfer before commencing the procedures described below. All washes were carried out in phosphate-buffered saline

TABLE 1 | Fetal and neonatal outcomes.

	115 d GA		124 d GA		1-day-old lamb	
	Control	**FGR**	**Control**	**FGR**	**Control**	**FGR**
Number of ewes	6		5		8	7
Number of fetuses	6	6	5	5	8	7
GA at surgery	107 ± 1.0	107 ± 1.0	104 ± 0.6	104 ± 0.8	105 ± 1.2	105 ± 0.9
Male:female	4:2	4:2	2:3	2:3	4:4	3:4
Body weight (kg)	1.9 ± 0.2	1.6 ± 0.1	3.5 ± 0.1	2.5 ± 0.2*	4.8 ± 0.5	3.5 ± 0.5*
Brain weight (g)	34.3 ± 1.6	34.4 ± 1.9	48.3 ± 2.0	43.9 ± 1.6	55.6 ± 2.7	51.3 ± 2.7
Brain:body weight (g/kg)	18.1 ± 1.3	21.8 ± 2.2	13.8 ± 0.5	17.0 ± 1.1*	11.8 ± 0.4	16.1 ± 1.8*
Blood gas parameters (average across gestation)						
Arterial oxygen saturation (%)	59 ± 1.3	52 ± 1.3*	70 ± 0.8	58 ± 0.8*	62 ± 1.5	43 ± 2.0*
Cerebellum						
Cross-sectional area (mm^2)	$1.3 \times 10^5 \pm 1.2 \times 10^4$	$1.3 \times 10^5 \pm 1.1 \times 10^4$	$1.6 \times 10^5 \pm 1.6 \times 10^4$	$1.8 \times 10^5 \pm 1.6 \times 10^4$	$2.1 \times 10^5 \pm 1.9 \times 10^4$	$2.2 \times 10^5 \pm 1.3 \times 10^4$
Cross-sectional area:body weight (mm^2/kg)	$7.4 \times 10^4 \pm 1.0 \times 10^4$	$8.1 \times 10^4 \pm 6.0 \times 10^3$	$4.7 \times 10^4 \pm 4.7 \times 10^3$	$7.1 \times 10^4 \pm 1.0 \times 10^4$	$4.7 \times 10^4 \pm 4.7 \times 10^3$	$6.2 \times 10^4 \pm 4.3 \times 10^{3*}$
EGL width (Mm)	22.0 ± 1.6	29.6 ± 2.6*	21.7 ± 2.3	21.5 ± 3.3	14.1 ± 0.7	14.0 ± 0.9
ML width (mm)	142 ± 6	112 ± 5*	160 ± 12	150 ± 11	173 ± 8	195 ± 5*
WM:cross-sectional area (mm^2)	0.1 ± 0.01	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.02	0.2 ± 0.02	0.2 ± 0.01
The number of Purkinje cells (mm)	0.016 ± 0.002	0.016 ± 0.001	0.015 ± 0.001	0.013 ± 0.001	0.015 ± 0.001	0.016 ± 0.001

Mean ± SEM are presented for each group.$p \leq 0.05$ vs. control.*
d GA, days gestational age; FGR, fetal growth restriction; EGL, external granular layer; ML, molecular layer; WM, white matter.

(PBS, 0.1 M, pH 7.4). For each immunohistochemical protocol, duplicate sections of the cerebellum from all treatment groups were included in a single run to eliminate variations between runs.

Glial fibrillary acidic protein (GFAP) was used to visualize astrocytes and astrocytic end-feet. Ionized calcium binding adaptor molecule 1 (Iba-1) was used to identify microglia. The DNA adduct 8-hydroxy-2-deoxyguanosine (8-OHdG) was used as a marker of oxidative DNA damage. NEUronal nuclei, clone A60 (NeuN), was used to identify neuronal nuclei; microtubule-associated protein 2 (MAP-2) was used to examine neuronal filaments, and albumin was used to detect blood protein extravasation. GFAP, Iba-1, 8-OHdG, NeuN, MAP-2, and albumin were identified using the following: monoclonal mouse anti-GFAP (1:400; Sigma-Aldrich), rabbit anti-Iba1 (1:1,000; Wako Pure Chemical Industries Ltd., Osaka, Japan), mouse anti-NeuN (1:200; Millipore Corporation, USA), mouse anti-MAP-2 (1:200; Thermo Fisher Scientific, Waltham, Massachusetts, USA), and rabbit anti-sheep albumin (1:1,000; Accurate Chemical and Scientific Corp., Westbury, NY, USA), respectively. Antibodies were diluted in PBS. All sections were treated with a secondary antibody (1:200; biotinylated anti-rabbit or anti-mouse IgG antibody; Vector Laboratories, Burlingame, CA, USA) and immunolabeling was visualized using 3,3-diaminobenzidine (DAB; Pierce Biotechnology, Rockford, IL, USA). Positive and negative control sections were included in each run. Sections were viewed at 400× magnification using a light microscope (Olympus BX-41, Japan).

Perls' Prussian Blue

Perls' staining was used to observe the presence of microbleeds (which presented as free iron) within the cerebellum. Following dewaxing, the slides were rehydrated in distilled water for 5 min. While this took place, a solution of equal parts 4% aqueous potassium ferrocyanide and 4% hydrochloric acid was mixed, and then heated to 60°C in a microwave that was placed within a fumehood to prevent exposure to hydrogen cyanide fumes. Slides were then placed into the heated solution for 40 min and then allowed to cool. The sections were rinsed in running distilled water for 2 min and then placed into a dish and counter-stained with Nuclear Fast Red for 4 min. Following this, the slides were rinsed for another 2 min under running distilled water, and then dehydrated in ascending alcohol concentrations, cleared in xylene, and cover-slipped. Sections were examined for the presence of microbleeds using a light microscope (Olympus BX-41, Japan). All areas of the cerebellum were examined, and bleeds were counted manually.

Image Analysis

Densitometric calculations for GFAP, 8-OHdG (internal granular layer; IGL), and MAP-2-positive immunoreactivity was performed using a computerized image analysis system (ImageJ version 10.2, NIH, Bethesda, Maryland, USA) that reads optical density as gray levels, at 400× magnification. An investigator (TY), blinded to the experimental groups, selected the threshold levels of detection, where nonspecific background was identified and the threshold level was then kept constant for all images within

a given immunohistochemistry run. For cell counts and scoring, two sections of the cerebellum per animal were examined, and the number of immunopositive cells for 8-OHdG [molecular layer (ML) and WM] and NeuN, or percentage area occupied by Iba-1-positive cells, was calculated using an average of three fields of view per area, and the results were averaged across the animals in each group. Analysis for cresyl violet and acid fuchsin staining, Purkinje cells, IGL and ML width, GFAP, 8-OHdG, Iba-1, MAP-2, and NeuN immunohistochemistry was performed in the anterior and posterior lobules (II, VI, and VIII), in three randomly selected fields of view. Analysis for microbleeds and albumin extravasation was performed on the entire section of the cerebellum, including all folia. The number of incidences for microbleeds and albumin extravasation was counted and averaged per section in duplicate across all animals in each group. Neuronal cell morphology was assessed using cresyl violet and acid fuchsin staining; cells that displayed swelling of organelles, loss of cell wall, and/or nuclear membrane integrity were considered abnormal. The cross-sectional area of the entire cerebellum and the WM were performed on the cresyl violet and acid fuchsin-stained sections using Image J to trace around each area, and data for cerebellar WM are presented per total area. The IGL and ML width were measured using the same program and data presented as μm (**Figure 1**).

Data Analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software, San Diego, United States). An unpaired nonparametric *t*-test was performed to compare between the control and FGR group at each gestational age. Significance was accepted when $p \leq 0.05$.

RESULTS

We induced late-onset placental insufficiency in fetal sheep (refer to **Table 1** for *n* numbers), which resulted in chronic fetal hypoxia that worsened compared to control fetuses over the course of gestation; 115 d GA 52 vs. 59% FaO_2, 124 d 58 vs. 70%, and 142 d GA 43 vs. 62% FaO_2 (**Table 1**). The success rate for fetal survival after FGR surgery was 83%, with all fetal deaths occurring within the first 24 h post-surgery. Body weights were reduced in FGR fetuses compared to age-matched controls, and this was statistically significant at 124 d GA and in 1-day-old lambs (**Table 1**). Brain weight was spared in FGR fetuses and lambs, such that the brain to body weight ratio was significantly increased at 124 d GA and in 1-day-old FGR lambs. The total area of the cerebellum to body weight ratio was also significantly increased in the FGR 1-day-old lambs compared to controls. FGR had no effect on the area of cerebellar WM (**Table 1**).

Morphological Changes in the Cerebellum

We first assessed basic cerebellar morphology in the control animals. Cresyl violet and acid fuchsin staining showed that, at 115 d GA, a prominent external granular cell layer (EGL) was apparent in control brains (**Table 1**), which actively proliferates over the preterm period studied, wherein the cells migrate inward to form the IGL (Volpe, 2009). After 124 d GA, the EGL was less prominent, and was further decreased in width in 1-day-old control lambs (EGL at 115 d: GA 22.0 ± 1.6 μm, at 124 d: GA 21.7 ± 2.3 μm, and in 1-day-old lambs: 14.1 ± 0.7 μm). By contrast, the width of the ML in control animals increased as gestation progressed (**Table 1**). At 115 d GA, the width of the EGL in FGR fetuses was significantly thicker than control fetuses (**Figures 1A,B**), but this difference was no longer seen at 124 d GA and in 1-day-old lambs. In FGR animals compared to control animals, the width of the ML was significantly reduced at 115 d GA; no difference was observed at 124 d GA, but then became significantly wider in 1-day-old lambs (**Figures 1C,D**).

At 115 d GA, Purkinje cell bodies were evident in control brains, and the number of Purkinje cells remained relatively consistent from 115 d GA through to 1-day-old postnatal lambs (**Table 1**). The IGL had a dense population of neurons, and the boundaries between IGL and the WM were also apparent at 115 d GA. Qualitative analysis revealed that the population of cells in the IGL appeared to be increased by 124 d GA and even further in 1-day-old lambs, and sharp boundaries were formed between the IGL and WM, such that the two layers were highly distinguishable.

There was no evidence of gross lesions within the FGR brains, but at 115 d GA, FGR fetuses showed evidence of neurons with abnormally shaped, darkly stained nuclei, vacuolization, and patchy coverage in parenchymal WM tissue (**Figure 1F**); this was also evident in FGR fetuses at 124 d GA and in 1-day-old lambs. We noted that the cerebellar Purkinje cells exhibited altered cellular morphology and arrangement in FGR brains, as evidenced by intensely stained Purkinje cell bodies of abnormal shape and Purkinje cell disorganization, most apparent in 1-day-old lambs (**Figure 1H**).

We investigated the presence of microbleeds within the cerebellar tissue (**Figure 2A**), visualized as bright blue patches (Perls' positive staining), counter-stained with nuclear fast red (**Figures 2D,H,L**). Control fetuses did not exhibit any blue staining; (**Figures 2C,G,K**) however, microbleeds were present in 4/8 control lambs. In FGR brains, blue staining was present in 4/6 fetuses at 115 d GA, and 5/5 fetuses at 124 d GA, with microbleeds present within the ML, IGL, and WM. Microbleeds were also identified in 5/7 FGR lambs.

The absence of immunoreactive albumin within the brain is indicative of a functional BBB, and albumin was not observed within control fetal and lamb brains (**Figures 2B,E,I,M**), with the exception of albumin extravasation in one of five control animals at 124 d GA. In FGR brains, the occurrence of albumin extravasation from blood vessels (BVs) was observed in all regions of the cerebellum (**Figure 2B**). Where albumin was present in FGR brains, intense immunoreactivity was predominantly observed in brain parenchyma adjacent to BVs (**Figures 2F,J,N**).

Immunohistochemistry

GFAP-positive astrocytes were present in the cerebellar ML, GL, and the WM of control and FGR brain. At 115 d GA,

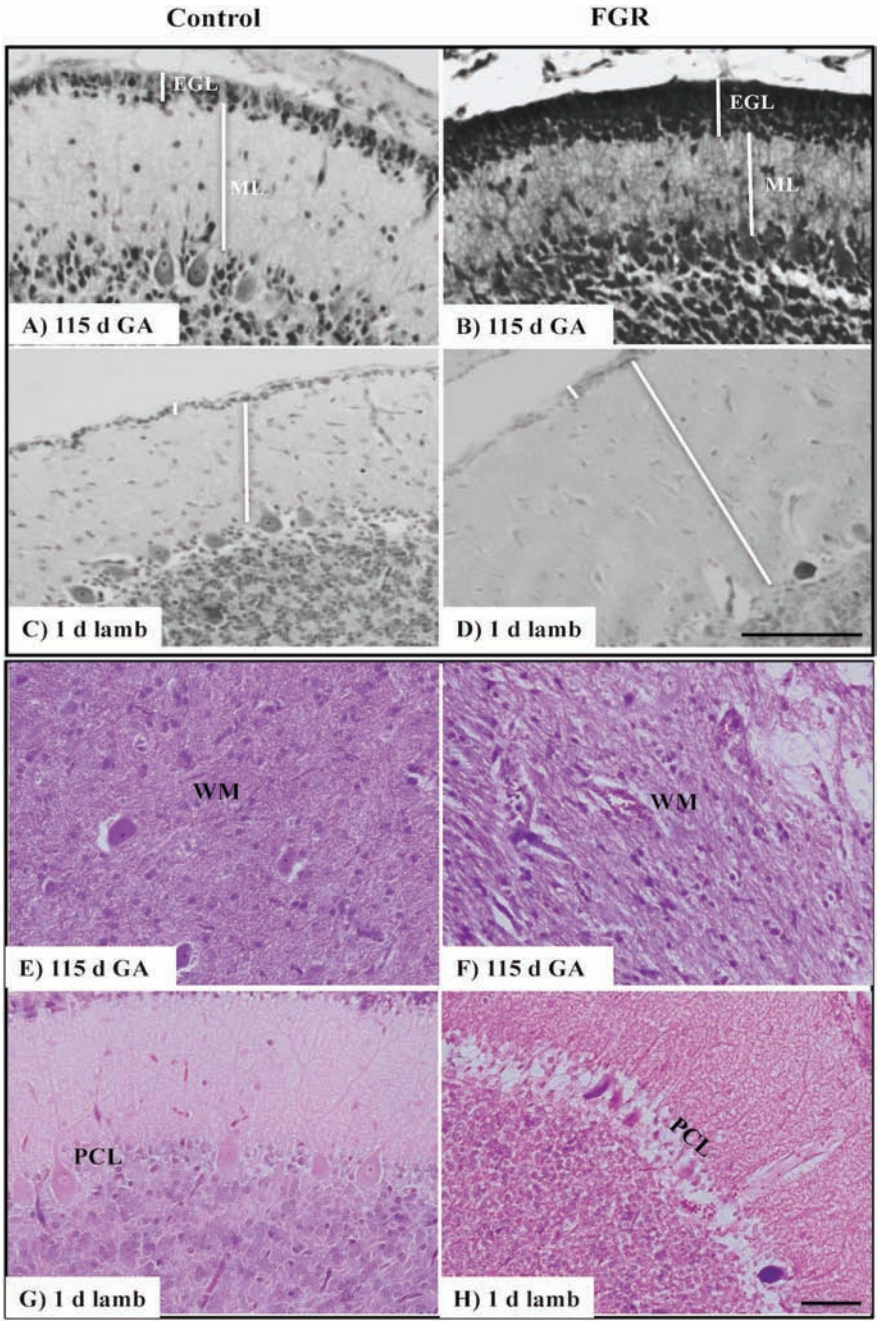

FIGURE 1 | Representative micrographs of cresyl violet and acid fuchsin staining in a 115 d GA fetus and 1-day-old lamb of control **(A,C,E,G)** and FGR **(B,D,F,H)** brain. **(A–D)** show measurements of EGL and ML on each image. Within FGR brains, note the presence of vacuolization in parenchymal WM tissue **(F)** and Purkinje neurons with abnormally shaped, darkly stained nuclei **(H)**. EGL, external granular layer; ML, molecular layer; PCL, Purkinje cell layer; WM, white matter. Scale bar for **(A–D)** in **(D)** = 100 μm and **(E–H)** in **(H)** = 50 μm.

astrocytes were not affected by FGR. However, the density of the astrocytic cell bodies and processes appeared to be reduced in all FGR brains at 124 d GA and 1-day-old lambs compared to control animals but this was only statistically significant in the IGL and WM at 124 d GA (**Figures 3A–C**). Furthermore, we observed very few end-feet of astrocytes associated with BVs in FGR animals (**Figures 3D,E**). The association of astrocytic end-feet to BVs is critical for maintenance of the BBB, and therefore, in this situation, a decrease in astrocyte density and end-feet attachment has likely compromised the BBB in FGR offspring.

We quantified microglial cells, and found that in FGR fetuses at 124 d GA, there was an overall increase in the percentage area coverage of microglia that was significant in the GL and

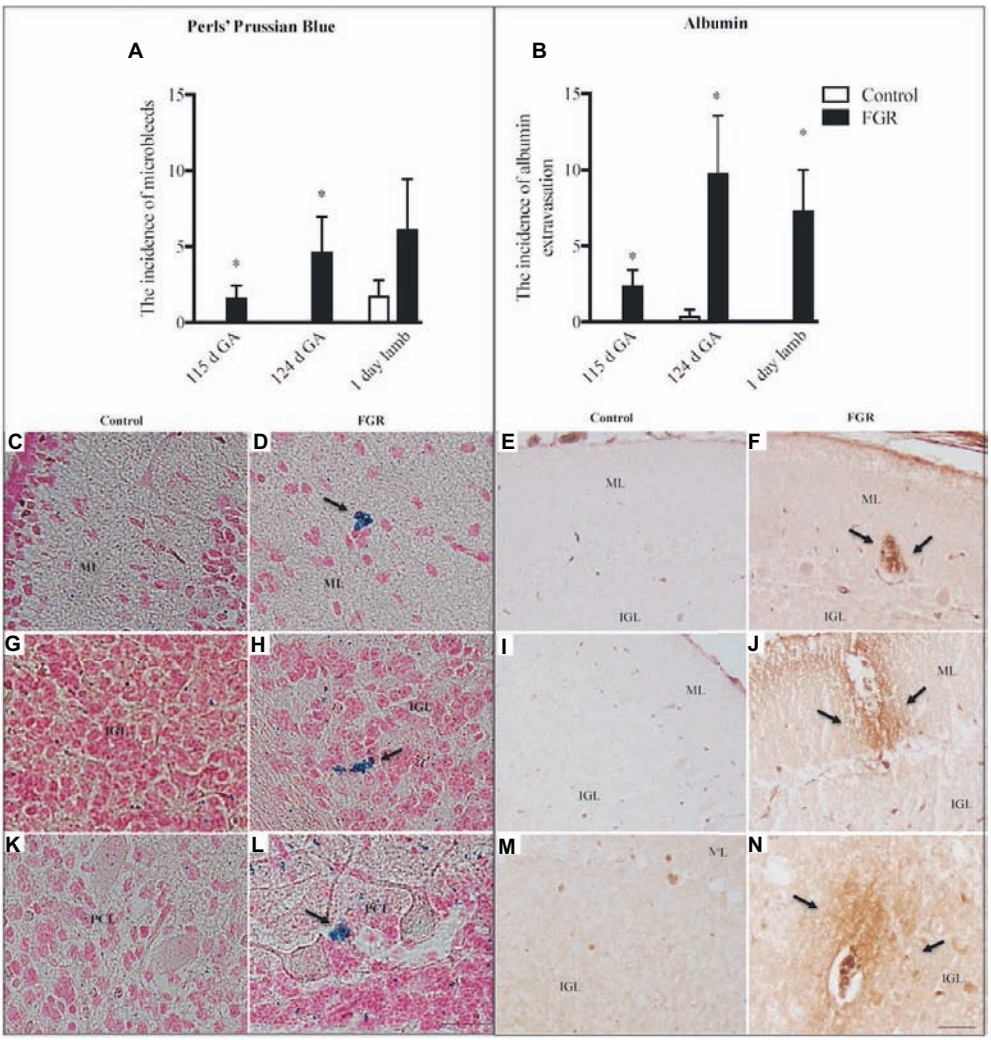

FIGURE 2 | The number of microbleeds **(A)** and albumin extravasation from blood vessels (BVs) **(B)** in the cerebellum of the fetal brain at 115 d GA, 124 d GA, and 1-day-old lamb of control and FGR animals. Each bar represents the mean ± SEM; $^*p \leq 0.05$ versus control. Representative micrographs showing Perls' Prussian Blue staining and albumin immunohistochemistry in a 115 d GA fetus **(C–F)**, 125 d GA **(G–J)**, and 1–day-old lamb **(K–N)** brains. Microbleeds were visualized as bright blue patches that were counter-stained with nuclear fast red. Arrows indicate the sites of microbleeds/albumin extravasation. FGR, fetal growth restriction; PCL, Purkinje cell layer; ML, molecular layer; IGL, internal granular layer. Scale bar for Perls in **(L)** = 25 μm. Scale bar for albumin in **(N)** = 50 μm.

WM (**Figures 4A–C**). There was no difference in microglial cell coverage in FGR (**Figure 4H**) and control (**Figure 4G**) animals at 115 d GA and 1-day-old lambs.

We next examined whether oxidative stress was upregulated in the FGR cerebellum. Cells that were positive for 8-OHdG immunoreactivity were present in control and FGR brains (**Figures 4I,J**). The number of cells that were positive for 8-OHdG was significantly increased in FGR 1-day-old lambs within the ML and WM compared to control brains (**Figures 4D–F**).

We examined neuronal cell number (NeuN) and the morphology of neuronal filaments (MAP-2) within the cerebellum. We utilized NeuN as a neuronal-specific nuclear protein (Mullen et al., 1992) and, as expected, NeuN-positive cells were localized to the cerebellar IGL and not the ML and WM, which is consistent with published data (Zimatkin and Karniushko, 2016), indicative that we had appropriately labeled for mature IGL neurons. The number of mature neurons was significantly reduced in FGR brains of 1-day-old lambs within the IGL compared to control lambs (**Figures 5A,C,D**). When we examined the expression of the MAP-2-positive immunoreactivity within the ML, a region where neuronal filaments extend to other areas in the cerebellum, we observed an overall decrease in the expression of MAP-2 at all gestational ages in the FGR animals, but this decrease was statistically significant at 115 d GA and 1-day-old lambs, compared to control (**Figures 5B,E,F**).

DISCUSSION

Here, we examined the effects of placental insufficiency, chronic fetal hypoxia, and FGR on cerebellar development at three timepoints corresponding to the last trimester of human

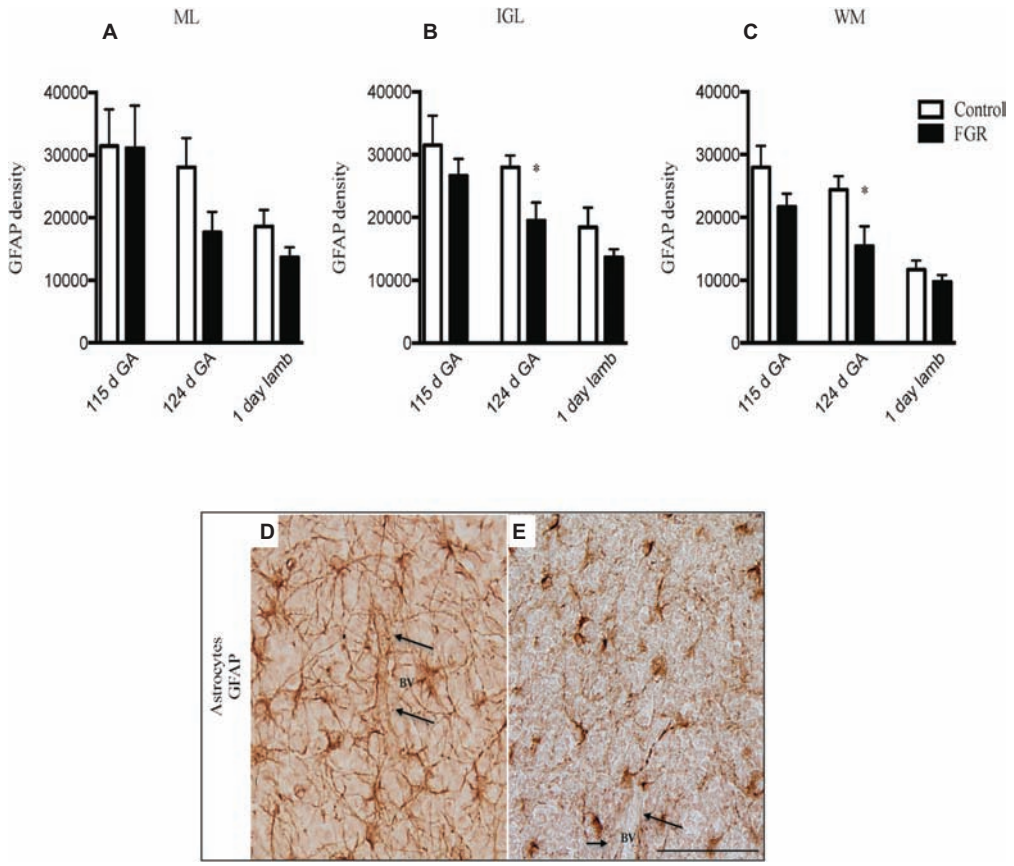

FIGURE 3 | Glial fibrillary acidic protein (GFAP) density in the molecular layer [ML **(A)**], internal granular layer [IGL **(B)**], and white matter [WM **(C)**] in the fetal brain at 115 d GA, 124 d GA, and 1-day-old lamb of control and FGR animals. Comparisons are made within the same brain region at the same gestation and not across regions or across gestation. Each bar represents the mean ± SEM; $^*p \leq 0.05$. Representative micrographs showing GFAP-positive immunoreactivity in the fetal IGL at 124 d GA of control **(D)** and FGR **(E)** brain. Arrows pointing to a blood vessel (BV), showing the damaged astrocytic end-feet. FGR, fetal growth restriction. Scale bar = 50 μm.

pregnancy and into the neonatal period. We show that cerebellar growth and development are adversely affected soon after the onset of hypoxia, and that chronic fetal hypoxia leads to a progressively altered developmental profile of the cerebellum. This is the first study to characterize maturation of normal and abnormal morphology within cerebellum from appropriately grown and growth-restricted fetuses and neonates. Most strikingly, newborn FGR lambs demonstrated neuropathology within the cerebellum after birth, with a significant, ~18% decrease, in the number of granule cell bodies within the IGL and an ~80% reduction in neuronal extension and branching within the ML. The foundations of neonatal cerebellar neuropathology were laid down soon after the onset of placental insufficiency and fetal hypoxia. The structural integrity of neurons was already aberrant in the FGR cerebellum at 115 d GA, and by 124 d GA, inflammatory cells were upregulated and the BBB was compromised. These neuropathologies occurred in the absence of gross cerebellar abnormalities; the cross-sectional area of the whole cerebellum and the WM were not different in size compared to control, and no cystic lesions were present in the brain of FGR offspring. Thus, we show that chronic *in utero* compromise leads to progressive maturational deficits in the cerebellum of FGR offspring, likely to have significant functional implications.

We induced late-onset placental insufficiency to mimic the clinical situation of FGR that is most commonly observed (Baschat, 2014). This ovine model of late-onset FGR did not significantly affect body weight 10 days after induction of placental insufficiency (115 d GA); however, body weight was significantly reduced in FGR offspring at 19 days (124 d GA) and 43 days (1-day-old lamb) following the onset of placental dysfunction. This was accompanied by a significant increase in brain to body weight ratio, a finding that supports our previous work (Miller et al., 2009, 2014) and reflects asymmetric growth in response to chronic hypoxia (Baschat, 2014; Miller et al., 2016).

It was interesting to note that, while body weight was not yet affected at 115 d GA, FGR fetuses were already experiencing significant hypoxia, and this was associated with early signs of cerebellar neuropathology. At 115 d GA, many cerebellar granule cells in FGR animals were abnormally shaped, with darkly stained nuclei – not observed in control fetuses – and similar cellular abnormalities remained evident at 124 d GA and in 1-day-old lambs. This supports previous work showing that both acute and chronic hypoxia result in cerebellar

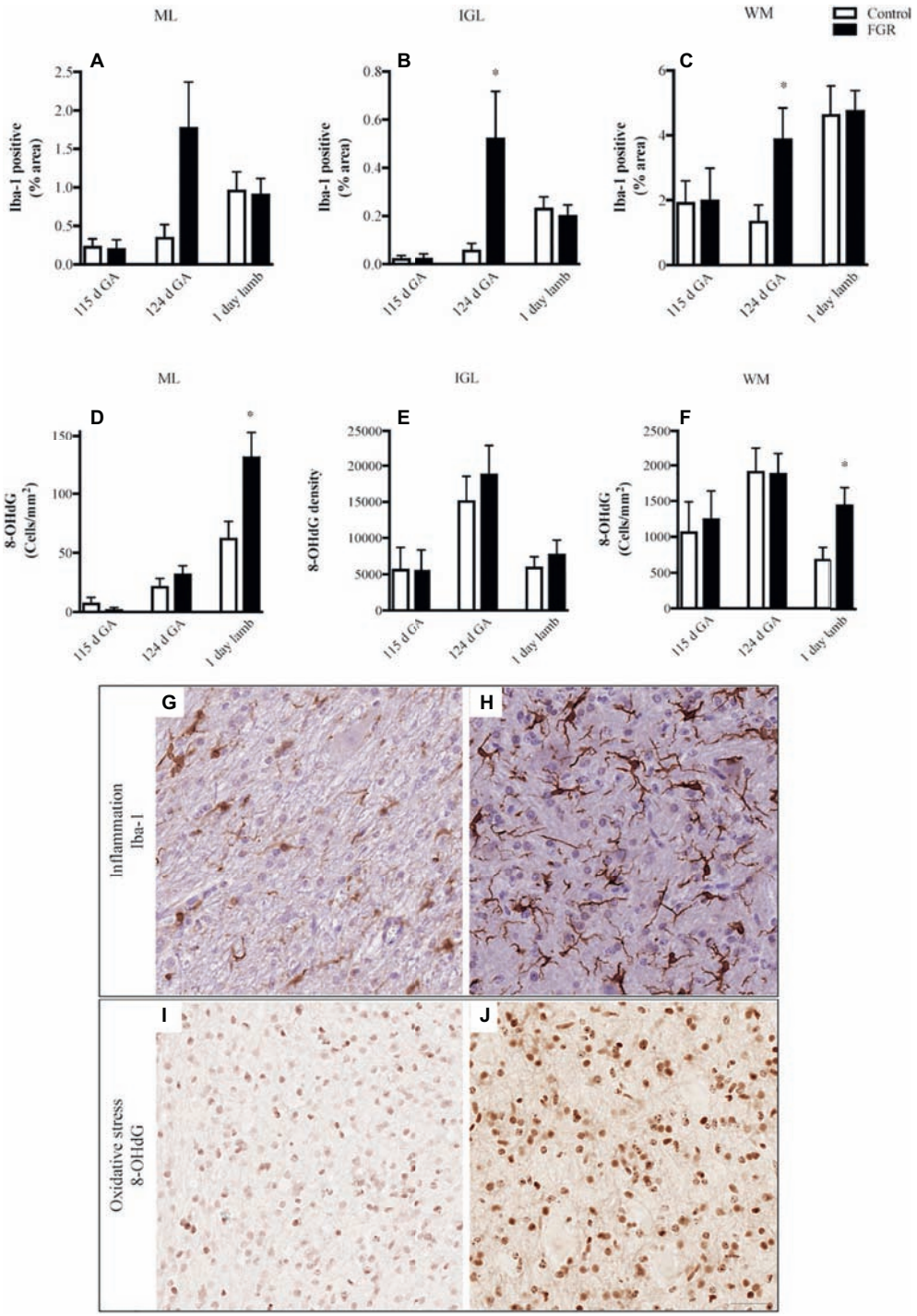

FIGURE 4 | The number of Iba-1-positive cells **(A–C)** and 8-hydroxy-2-deoxyguanosine (8-OHdG)-positive cells **(D–F)** in the molecular layer (ML), internal granular layer (IGL), and white matter (WM) in the fetal brain at 115 d GA, 124 d GA, and 1-day-old lamb of control and FGR animals. Each bar represents the mean ± SEM; *$p \leq 0.05$ versus control. Representative micrographs showing Iba-1- **(G,H)** and 8-OHdG- **(I,J)** positive immunoreactivity in the WM in 124 d GA **(G,H)** and 1-day-old lambs **(I,J)**. Images from control **(G,I)** and FGR **(H,J)** animals are shown. FGR, fetal growth restriction. Scale bar = 25 μm.

neuropathology, as described in fetal sheep (Yawno et al., 2009), rats (McDougall et al., 2017), and guinea pigs (Tolcos et al., 2018); however, these studies did not examine the ontogeny of the cerebellum. Structurally, at 115 d GA, the width of the EGL in FGR animals was significantly increased, and this was associated with a significant decrease in the width of the ML compared to control animals. The changes in the EGL and ML were resolved at 124 d GA. However, in 1-day-old FGR

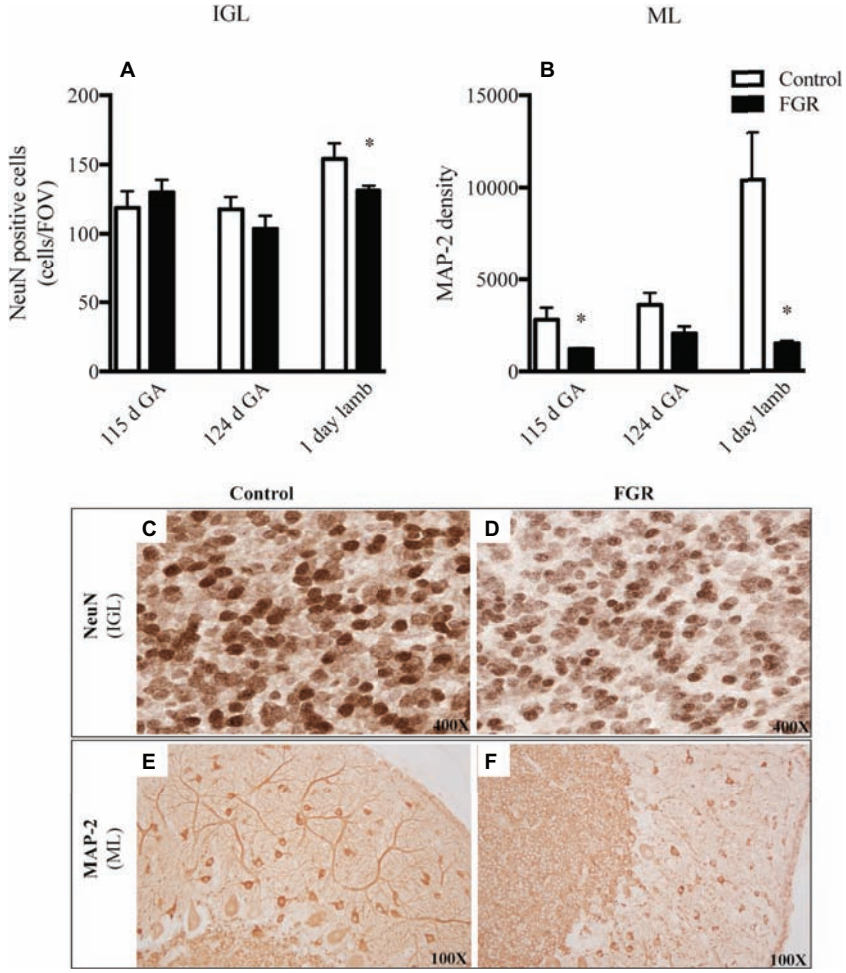

FIGURE 5 | The number of NeuN-positive cells per field of view **(A)** and MAP-2 density **(B)** in the internal granular layer (IGL) and molecular layer (ML) in the fetal brain at 115 d GA, 124 d GA, and 1-day-old lamb of control and FGR animals. Each bar represents the mean ± SEM; $^*p \leq 0.05$ versus control. Representative micrographs showing NeuN-positive immunoreactivity in the IGL **(C,D)** and MAP-2 in the ML **(E,F)** in 1-day-old lambs. Images from control **(C,E)** and FGR **(D,F)** animals are shown. FGR, fetal growth restriction.

lambs, the width of the ML was significantly increased compared to control lambs. These findings support previous results from other models of FGR (McDougall et al., 2017; Tolcos et al., 2018), where it was suggested that the increase in EGL width maybe due to impaired granule cell migration out of the EGL, increased cell proliferation, and reduced granule cell apoptosis. Even if these structural changes were corrected at 124 d GA, alterations were still evident in 1 day-old FGR lambs, which could lead to changes in cellular connectivity and cerebellar circuitry. Hence, further investigations on cell migration, proliferation, and apoptosis are warranted.

We noted that Purkinje cell bodies were morphologically abnormal, and the Purkinje cell layer was highly disorganized in 1-day-old FGR lambs only, thereby suggesting that progression of neuropathology over the course of late gestation had a direct effect on the Purkinje cell layer. Reduction in Purkinje cell number has been reported in the cerebellum of growth restricted guinea pigs and was associated with neurological and behavioral deficits postnatally (Mallard et al., 2000). While we did not see a change in the total number of Purkinje cell in our FGR offspring, it is known that Purkinje cells receive excitatory inputs from injured granule cells within the IGL (Sarna and Hawkes, 2003), thereby potentially making them more susceptible to abnormal development and injury.

A notable observation in the current study was the susceptibility of the cerebellum of FGR animals to hemorrhage. More than 80% of FGR fetuses (both 115 d and 124 d) showed evidence of brain bleeds, compared to no bleeds within control fetuses. In addition, we observed the presence of immunoreactive albumin adjacent to BVs within the brains of all FGR animals (115 d, 124 d, and 1-day-old lambs). This result is likely due to altered cerebrovascular development and suboptimal integrity of the BBB. We have previously examined cerebrovascular development within the subcortical and periventricular WM of the cerebrum, and shown specific cerebrovascular deficits within the FGR brain that include decreased basal lamina area,

reduced vascular endothelial growth factor (VEGF), fewer endothelial cells, and reduced BBB integrity (Castillo-Melendez et al., 2015, 2017). The structural and functional features of the BBB are normally maintained by the contribution and cellular interactions of the endothelial cells, pericytes, and astrocytic end-feet contacts (Kim et al., 2013). In particular, close astrocyte association with BVs is important in development as it provides functional and structural support by controlling inflammatory cell infiltration and maintaining homeostasis (Farhy-Tselnicker and Allen, 2018). Thus, our finding that the FGR brain showed reduced expression of astrocytic density from 124 d GA potentially plays a critical role in abnormal brain development. In addition, with reduced glial support, neuronal function may be even further compromised (Laming et al., 2000). It is worth noting that the presence of microbleeds (Perls' stain) in our control lamb brains was unexpected; we are unsure to why this has happened; however, all lambs in this cohort were delivered naturally which could induce temporary instability of the BBB *via* hypoxia at birth, but it is also important to note that the albumin data show that control lambs did not show any albumin extravasation and an indication of a more stable BBB.

Concomitantly with altered BBB function at 124 d GA, we also saw a neuroinflammatory response within the cerebellum of FGR fetuses in this age group, with a large increase in microglial cells in all cerebellar regions examined, compared to control brains. This finding fits with growing knowledge of the contribution of neuroinflammation toward neuropathology in FGR animal studies (Wixey et al., 2018) and in human preterm infants (Buser et al., 2010). Inflammation-induced brain injury is associated with WM damage, acute BBB dysfunction, and neurovascular unit injury, and in turn linked with behavioral alterations (Stolp et al., 2011). It is interesting to note that an elevation in neuroinflammation was only observed within the FGR brain at 124 d GA, and this was linked with an altered astrocyte profile at this age, and high susceptibility to hemorrhage. Previous work has shown that injured glial cells release factors that act on target cells to initiate responses of activated immune cells in the periphery, in turn leading to inflammatory cell infiltration of tissues. These events usually occur when there has been destruction or compromise of the BBB (Streit et al., 1998; Sroga et al., 2003). Taken together, these results are indicative that this developmental timepoint (roughly equivalent to 34 weeks in human cerebellar development) is particularly vulnerable for BBB dysfunction linked with upregulation of neuroinflammation. It is of interest to note that clinically, rates of intraventricular hemorrhage (IVH) are not increased in FGR infants born preterm at 28 weeks compared to appropriately grown infants, but IVH rates are significantly elevated in human infants born late preterm (>34 weeks) (Gilbert and Danielsen, 2003; Ortigosa Rocha et al., 2010), indicative of the vulnerability of the BBB in late gestation (Wixey et al., 2018). It is, however, worth noting that the marker we used to identify inflammatory cells does not discriminate between resting and activated microglia. Therefore, it would be of interest to further phenotype the inflammatory cells to examine whether late-onset FGR induces a pro-inflammatory environment within the cerebellum, which may be amenable to treatment.

We examined the presence of oxidative DNA damage (8-OHdG) to indicate whether oxidative stress was upregulated in the cerebellum of FGR animals and contributed to the neuropathology observed (Yawno et al., 2017). During fetal gestation, both control and FGR animals showed cellular oxidative stress to be present within the cerebellum, with no differences seen between the groups. In 1-day-old lambs, oxidative cell damage was significantly upregulated within the cerebellar ML and WM of FGR offspring, compared to control animals. All lambs were born naturally at term and cared for by their mother after birth, with no need for intervention or supplemental oxygen. Therefore, the observation of increased oxidative damage within the brain of newborn FGR lambs may represent a response to birth. Potentially, this is contributed by relative hyperoxia experienced by FGR lambs after birth compared to control lambs, given their *in utero* conditions of chronic hypoxia. It may also be due to the reduced anti-oxidant defense capabilities in the FGR lamb. We have previously shown that in FGR offspring, circulating malondialdehyde concentration (a marker of oxidative stress) was elevated compared to appropriately grown offspring (Polglase et al., 2017) and that the same FGR offspring had significantly increased levels of oxidative stress within the brain, in both neuronal cell bodies of the cortex and fiber tracts within the periventricular WM (Miller et al., 2014). This is the first study to show that oxidative stress is upregulated in the FGR cerebellum, appearing to affect granule cell axons and migrating neurons within the ML. Oxidative DNA damage might be a precursor to cell death, which would require substantiation in long-term studies.

Mature IGL neurons (NeuN-positive cells) were significantly reduced in the neonatal FGR brains compared to control lambs but no difference was apparent at earlier timepoints, thereby suggesting that cerebellar neuropathology resulting from late-onset FGR takes time to manifest. This finding is in agreement with other literature showing a reduction in the density of cells undergoing mitosis in the cerebellar granular layer of hypoxemic fetal sheep compared to controls (Rees et al., 1997) and suggests that chronic hypoxia is causing an arrested maturational profile in IGL neurons or that mature neurons have not completed migration into the IGL. In addition to reduced total number of neurons in the granular layer, we also observed a decrease in the expression of MAP-2 across gestation in the ML of all FGR animals, indicative of disrupted dendritic branching. MAP-2 is critical for neurite extension and branching, and for cessation of cell division (Johnson and Jope, 1992). In rodents, it has been shown that loss of MAP-2 expression is associated with neuronal degeneration, which has short- (Kinugasa-Taniguchi et al., 2010) and long-term (Matesic and Lin, 1994) effects on cognition and anxiety-related behavior (Matesic and Lin, 1994). Fundamentally, loss of neurons and altered neuronal morphology of existing neurons may well underlie deficits in spatial motor function and coordination, which are present in children who were growth restricted at birth (Tolsa et al., 2004).

A noted limitation of the current study is that we did not have sufficient numbers of animals to examine potential sex differences. A recent review has described important female

versus male differences in fetal growth and sex-specific response to preterm birth and compromised placental function (Alur, 2019). In further studies, it would be of interest to examine cerebral and cerebellar development in males and females following compromised intrauterine environment, with the potential for tailored therapeutics.

Cerebellar injuries are often overlooked in experimental and clinical studies of brain neuropathology associated with acute or chronic hypoxia. Here, we demonstrate the progressive nature of developmental deficits in the cerebellum, associated with increasing placental insufficiency, chronic hypoxia, and FGR. These results suggest that the period of suboptimal *in utero* environment contributes to cerebellar pathology in late gestation fetal sheep. These results also indicate that late preterm age in sheep is a time of high risk for glial disturbances. Glial cells play an important function in the survival and maintenance of the neurovascular unit, and conversely, altered glial number or function can cause disruption to the BBB and brain developmental more broadly. We confirm that cerebellar injuries develop antenatally in FGR, and therefore, interventions to prevent long-term motor and coordination deficits should be implemented antenatally and/or perinatally, potentially targeting neuroinflammatory and oxidative stress pathways.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Monash Medical Centre Animal Ethics Committee A. The protocol was approved by the Monash Medical Centre Animal Ethics Committee A.

AUTHOR CONTRIBUTIONS

TY performed the experiments and data analysis, and wrote the original draft of the manuscript. GJ, MC-M, and SM provided funding, designed the experimental protocol, and helped draft the manuscript. AS assisted with animal experiments and data analysis, and helped draft the manuscript. YP assisted with data analysis.

FUNDING

The authors wish to acknowledge funding support from the Cerebral Palsy Alliance Australia and the Victorian Government's Operational Infrastructure Support Program.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 20 June 2019
doi: 10.3389/fendo.2019.00274

Postnatal Catch-Up Growth After Suspected Fetal Growth Restriction at Term

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Objective: The aim of this study was to study growth patterns of children born after suspected fetal growth restriction (FGR) at term and to compare the effect of induction of labor (IoL) and expectant management (EM), also in relation to neurodevelopmental and behavioral outcome at age 2.

Methods: We performed a 2 years' follow-up of growth of children included in the Disproportionate Intrauterine Growth Restriction Trial at Term (DIGITAT) study, a Randomized Controlled Trial (RCT) comparing IoL with EM in pregnancies with suspected FGR at term. We collected data on child growth until the age of 2 years. Standard deviation scores (SDSs) for height and weight were calculated at different ages. We assessed the effects of IoL compared with EM and the effects of a birth weight below or above the 3rd or 10th centile on catch-up growth. Target height SDSs were calculated using the height of both parents.

Results: We found a significant increase in SDS in the first 2 years. Children born after EM showed more catch-up growth in the first month [height: mean difference −0.7 (95% CI: 0.2; 1.3)] and weight [mean difference −0.5 (95% CI: 0.3; 0.7)]. Children born with a birth weight below the 3rd and 10th centiles showed more catch-up growth after 1 year [mean difference −0.8 SDS (95% CI: −1.1; −0.5)] and after 2 years [mean difference −0.7 SDS (95% CI: −1.2; −0.2)] as compared to children with a birth weight above the 3rd and 10th centiles. SDS at birth had the strongest effect on adverse neurodevelopmental outcome at 2 years of age.

Conclusion: After FGR at term, postnatal catch-up growth is generally present and associated with the degree of FGR. Obstetric management in FGR influences postnatal growth. Longer-term follow-up is therefore needed and should be directed at growth and physical health.

Clinical Trial Registration: www.ClinicalTrials.gov, identifier SRCTN10363217.

Keywords: intrauterine growth restriction, fetal growth restriction, catch-up growth, Disproportionate Intrauterine Growth Restriction Trial at Term, follow-up

OPEN ACCESS

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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *20 September 2018*
Accepted: *15 April 2019*
Published: *20 June 2019*

Citation:
van Wyk L, Boers KE, van Wassenaer-Leemhuis AG, van der Post JAM, Bremer HA, Delemarre FMC, Gordijn SJ, Bloemenkamp KWM, Roumen FJME, Porath M, van Lith JMM, Mol BWJ, le Cessie S and Scherjon SA (2019) Postnatal Catch-Up Growth After Suspected Fetal Growth Restriction at Term. Front. Endocrinol. 10:274. doi: 10.3389/fendo.2019.00274

INTRODUCTION

Catch-up growth is considered to be a process of compensatory accelerated growth after a period of poor growth intrauterine. Many studies have shown that growth-restricted children show catch-up growth in the first years after birth (1–6). Catch-up growth is recognized when a child shows accelerated growth, which is visualized as an upward crossing of its centiles in length or weight growth.

Long-term consequences of fetal growth restriction (FGR) and catch-up growth have been studied extensively in the last two decades. It is now generally accepted that low birth weight as a consequence of growth restriction is a risk factor for cardiovascular disease later in life (7–9). It however remains debatable whether this is caused by the catch-up growth itself or the underlying pathophysiology of FGR, by the (extent of) postnatal catch-up growth, or perhaps by a combination. Studies have shown that FGR intrauterine growth restriction itself is associated with an alteration of genetic programming, independent of Body Mass Index (BMI), related to metabolic and cardiovascular diseases (10). On the contrary, others have shown that it is the postnatal catch-up growth and accelerated weight gain that lead to the increased risk (11).

Wit et al. suggest that catch-up growth can be either *complete* or *incomplete* (12). Catch-up growth is considered to be complete in case a mean final height is reached close to the mean target height based on the height of both parents (12). In case of incomplete catch-up growth, the target height range will not be reached and is associated with increased neurodevelopmental problems when compared to children with complete catch-up growth (13–15).

In the Disproportionate Intrauterine Growth Restriction Trial at Term (DIGITAT) trial (16)—a multicenter randomized controlled trial—the effects of induction of labor (IoL) vs. expectant management (EM) in pregnancies suspected of intrauterine growth restriction at term were compared. Suspected intrauterine growth restriction was defined as fetal abdominal circumference below the 10th percentile, estimated fetal weight below the 10th percentile, flattening of the growth curve in the third trimester (as judged by a clinician), or the presence of all three factors.

Data were also collected of women who refused randomization but gave permission for follow-up. Pregnant women with a singleton fetus in the cephalic position, beyond 36 weeks of gestation, and suspected FGR were counseled for trial participation. Primary fetal and maternal outcomes were comparable. Gestational age at birth was lower in the induction group (mean difference –iff (95% CI: –I%fe to –o%fe as was birth weight (mean difference 130 g, 95% CI: –I%f to –o%f $P < 0.001$) when compared to the expectant monitoring group. Despite this difference in (absolute) weight in favor of the children in the group with expectant monitoring, more babies in the expectant group were severely growth restricted, with a birth weight below the third percentile (31 vs. 13%; difference –if%, 95% CI: –I%fe to –o%fer $P < 0.001$) (16).

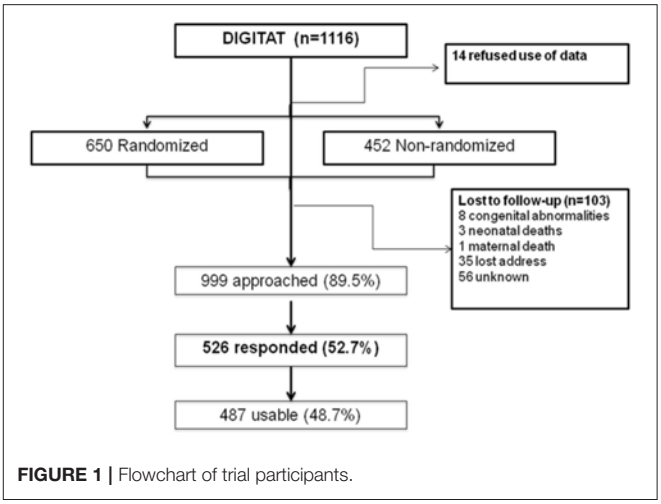

FIGURE 1 | Flowchart of trial participants.

At 2 years of age, a neurodevelopmental and behavioral follow-up study was performed and showed that children with low birth weights, especially if the birth weight was below the 3rd centile, had a greater risk [adjusted OR 3.6 (95% CI: 1.5–8.8)] for abnormal scores on the Ages and Stages Questionnaire (ASQ). The prevalence of these abnormalities was directly related to the severity of growth restriction (17).

The objective of the present study was to (1) analyze whether obstetric management (IoL vs. EM) policy influences catch-up growth, (2) assess whether children born in the DIGITAT trial (participants and nonparticipants) exhibit catch-up growth until age 2, and (3) assess long-term behavioral and neurodevelopmental outcomes in relation to catch-up growth.

METHODS

Participants

The study population consisted of children born to mothers who participated in the DIGITAT trial (16). During the study period, 1,116 women were eligible for the trial, of whom 14 refused participation, 452 women refused randomization but gave permission for use of their medical data and for follow-up, and 650 women were randomized to either induction of labor (IoL) or an expectant management (EM) (**Figure 1**). Both the randomized and nonrandomized women are included in this study.

Baseline Characteristics

Baseline characteristics of the mothers and children were collected at study inclusion and at birth and have been described elsewhere (16).

Abbreviations: ASQ, Ages and Stages Questionairre; CBCL, Child Behavior Checklist; CI, confidence interval; DIGITAT, Disproportionate Intrauterine Growth Restriction Trial at Term; IQR, interquartile range; FGR, fetal growth restriction; SD, standard deviation; SDS, standard deviation score.

Procedure

We collected growth data until the age of 2 of children born to mothers of the DIGITAT trial. General health, height, weight, and developmental milestones of children in the Netherlands are assessed regularly and free of charge in a national program at the Child Health Center's periodically. Parents were asked to participate in a postal inquiry and to send us the recorded growth data (height and weight) of their child until the age of 2 years and to report the height of the father. Maternal height was already recorded at baseline. Standard deviation scores (SDSs) or *Z* scores were calculated for height, weight, and weight for height at different ages based on, respectively, calculated target height- and population-based growth curves (18). Head circumference was not recorded.

Neurodevelopmental and behavioral outcomes of these children were also assessed at 2 years of age using two standardized parental questionnaires: the Ages and Stages Questionnaire (ASQ) (19) and the Child Behavior Checklist (CBCL) (20). The mean scores of both questionnaires were compared between groups,

TABLE 1 | Baseline characteristics.

Characteristic	Respondent ($n = 526$)	Non-respondent ($n = 473$)	Difference in % or mean (95% CI)
Maternal age (years)	29.7 (26.3; 33.8)	27.7 (23.3; 31.9)	2.2 (1.5; 2.8)**
Maternal Body Mass Index at study entry†	21.8 (19.7; 24.6)	21.8 (19.5; 24.8)	0.04 (−0.6; 0.7)
Maternal smoking in pregnancy§	134 (25.5)	201 (44.7)	−17.1 (−23.2; −11.1)**
Caucasian‡	448 (91.1)	354 (73.9)	17.2 (12.5; 21.8)**
EDUCATION			
Lower professional	200 (69.9)	243 (81.8)	−11.9 (−18.8;−5.0)**
Higher professional	86 (30.1)	54 (18.2)	11.9 (5.0; 18.8)**
Gestational age at birth	272.3 (265.6; 280.4)	272.5 (264.6; 280.8)	−0.02 (−1.2; 1.2)
Male sex	186 (38.9)	198 (38.4)	−0.4 (−5.673, 6.454)
Birth weight (grams)	2,505 (2,215; 2,771)	2,505 (2,281; 2,769)	30.0 (−77.4; 19.2)
Birth weight <10th centile	382 (72.7)	336 (71.1)	1.5 (−4.0; 7.2)
Birth weight <3rd centile	121 (25.4)	129 (25.2)	0.2 (−5.2; 5.7)
Composite adverse neonatal outcome	30 (5.7)	24 (5.1)	0.6 (−2.2; 3.4)

***p < 0.001. CI, confidence interval.*
Table shows median [IQR: 25th to 75th percentile) or number (%)].
Data were compared using the Student's t-test, chi-square, or Fisher's exact test.
† n = 457 for respondents; n = 394 for non-respondents.
§n = 485 for respondents; n = 449 for non-respondents.
‡ n = 492 for respondents; n = 479 for non-respondents.

TABLE 2 | Baseline characteristics of randomized participants (induction vs. expectant monitoring).

Characteristic	Induction ($n = 158$)	Expectant monitoring ($n = 134$)	Difference in % or mean (95% CI)
Maternal age (years)	28.03 (25.13; 32.07)	28.19 (24.69; 31.51)	−0.14 (−1.28; 0.99)
Maternal BMI at study entry†	22.1 (19.9; 25.8)	22.3 (20.2; 25.3)	−0.05 (−1.26; 1.15)
Maternal smoking in pregnancy§	34 (36.8)	43 (35.0)	0.02 (−0.1; 0.13)
Caucasian‡	12 (7.9)	13 (10.2)	−0.02 (−0.09; 0.05)
Education (higher professional)	16 (16.2%)	19 (22.9)	−0.07 (−0.18; 0.05)
Gestational age at birth	266.4 (261.5; 271.2)	277.5 (269.8; 283.6)	−9.91 (−11.85; −7.97)*
Male sex	57 (36.5)	53 (39.6)	− 3.1 (−8.0; 1.4)
Birth weight (grams)	2,435 (2,174; 2,660)	2,600 (2,230; 2,850)	−134 (−221; −46)*
Birth weight <10th centile	21 (13.3)	37 (27.8)	−14.5 (−24; −5.0)*
Birth weight <3rd centile	103 (65.2)	102 (76.7)	−11.5 (−22.0; −1.0)*
Composite adverse neonatal outcome&	7 (4.9)	7 (6.0)	−1.1 (−7; 4)

**p < 0.05.*
Table shows median [(IQR: 25th to 75th percentile) or number (%)].
Data were compared using the Student t-test, chi-square, or Fisher exact test.
† n = 141 for induction; n = 122 for expectant management.
§n = 144 for induction; n = 123 for expectant management.
‡ n = 151 for induction; n = 127 for expectant management.
& n = 143 for induction; n = 116 for expectant management.

TABLE 3 | Age, height, weight, and standard deviation score (SDS) for height, weight, weight for height, height SDS below target height, and difference between SDS in height and weight from birth.

Age (months)	Height (cm) (*n*)	Weight(g) (*n*)	SDS height	SDS weight	SDS weight for height (*n*)	SDS height-target height (*n*)	Difference in SDS height from birth (*n*)	95% CI	Difference in SDS weight from birth (*n*)	95% CI
Birth	46.4 ± 2.7 (236)	2,493 ± 397 (526)	−1.6 ± 1.3	−2.0 ± 0.86	NA	−1.4 ± 1.4	NA		NA	
1	50.7 ± 2.4 (370)	3,411 ± 476 (392)	−1.7 ± 1.1	−1.8 ± 1.0	−0.5 ± 1.1	−1.5 ± 1.2	0.2 (*n* = 161)	−0.4; 0.03	0.2 (*n* = 388)	0.1; 0.3;
3	57.7 ± 2.7 (419)	5,074 ± 685 (433)	−1.2 ± 1.1	−1.1 ± 1.0	−0.2 ± 1.2	−0.9 ± 1.1	0.4 (*n* = 188)	0.2; 0.6	0.9 (*n* = 429)	0.8; 1.0
6	64.7 ± 2.8 (439)	6,790 ± 825 (451)	−1.0 ± 1.1	−1.0 ± 1.1	−0.3 ± 1.1	−0.7 ± 1.2	0.6 (*n* = 202)	0.5; 0.8	1.1 (*n* = 446)	1.0; 1.2
9	69.9 ± 2.8 (355)	8,037 ± 937 (360)	−0.9 ± 1.1	−0.9 ± 1.1	−0.4 ± 1.0	−0.5 ± 1.0	0.8 (*n* = 159)	0.6; 0.9	1.1 (*n* = 356)	1.0; 1.2
12	72.8 ± 3.0 (296)	8,669 ± 1,103(301)	−0.8 ± 1.1	−1.0 ± 1.1	−0.5 ± 1.1	−0.5± 1.1	0.7 (*n* = 148)	0.5; 0.9	1.0 (*n* = 299)	0.9; 1.2
18	80.1 ± 3.3 (190)	10,101 ± 1,103 (192)	−0.9 ± 1.1	−1.1 ± −0.96	−0.8 ± 0.9	−0.5 ± 1.0	0.7 (*n* = 87)	0.4; 1.0	1.0 (*n* = 191)	0.8; 1.1
24	85.3 ± 4.2 (98)	11,362 ± 1,513 (100)	−0.9 ± 1.3	−1.0 ± 1.2	−0.7 ± 1.1	− 0.6 ± 1.2	0.6 (*n* = 43)	0.2; 1.0	0.9 (*n* = 99)	0.7; 1.2

Table shows mean (n) and standard deviation or 95% CI.

and the numbers of children with abnormal scores were also compared.

We found no commonly used or accepted definition of catch-up growth, and different studies use different definitions. We defined "complete catch-up growth" of height if the measured height SDS minus the target SDS was above −1.6. For weight and for weight for height, an SDS above −2 was used as an indication of complete catch-up growth (21).

Statistical Analysis

SDSs at birth were calculated using birth weight and height centiles, corrected for gestational age, using the formula of Niklasson (22). "Growth Analyser" v. 3.5 was used to calculate SDSs for height and weight at different ages using Dutch population-based curves (18). The formula of van Dommelen et al. (23) was used to calculate target height SDSs. This formula includes the height of both parents.

Continuous variables were presented as means with standard deviations (SDs), or medians with interquartile ranges (IQRs). Differences between groups were presented as differences in means or percentages with 95% confidence intervals (CIs). Continuous variables were compared using the Student's *t*-test or the nonparametric Mann–Whitney *U* test. The chi-square test and the Fisher's exact test were used for categorical variables.

We assessed whether catch-up growth was influenced by the following factors: IoL compared with EM (only for children included in the randomized study), the effects of birth weight below the 3rd or 10th centile, adverse neonatal outcome at birth (defined as 5-min Apgar score of <7, umbilical artery pH of <7.05, or admission to neonatal intensive care), birth weight centile, maternal smoking, breastfeeding, and gestational age at birth. Parents of the children included in the trial completed two questionnaires, the Ages and Stages Questionnaire (ASQ) and Child Behavior Checklist (CBCL), for presence of neurodevelopmental and behavioral problems, respectively.

The target height SD score was compared to the actual SD score using a paired *t*-test. Changes in SDS scores between different time points were calculated and compared between subgroups using non-paired *t*-tests. Repeated SDS measurements were analyzed using a linear mixed model, which accounts for missing values during follow-up with random person and age effects and fixed group effects. Since the relation between age and SDS scores was expected to be nonlinear, we used quadratic spline functions with knots on 1, 3, 6, 9, 12, and 18 months to model this relation. The results of these analyses are presented graphically.

We performed a logistic regression analysis to study the effect of gestational age and birth weight SDS. In a second analysis, we studied the additional effect of catch-up growth on the outcome. Multiple imputation was used to handle missing observations.

Analyses were carried out in Statistical Package for the Social Sciences (SPSS) 20. The repeated-measures analyses were carried out in R, version 3.02.

RESULTS

Baseline characteristics of respondents (completion of questionnaires) and non-respondents are shown in **Table 1**.

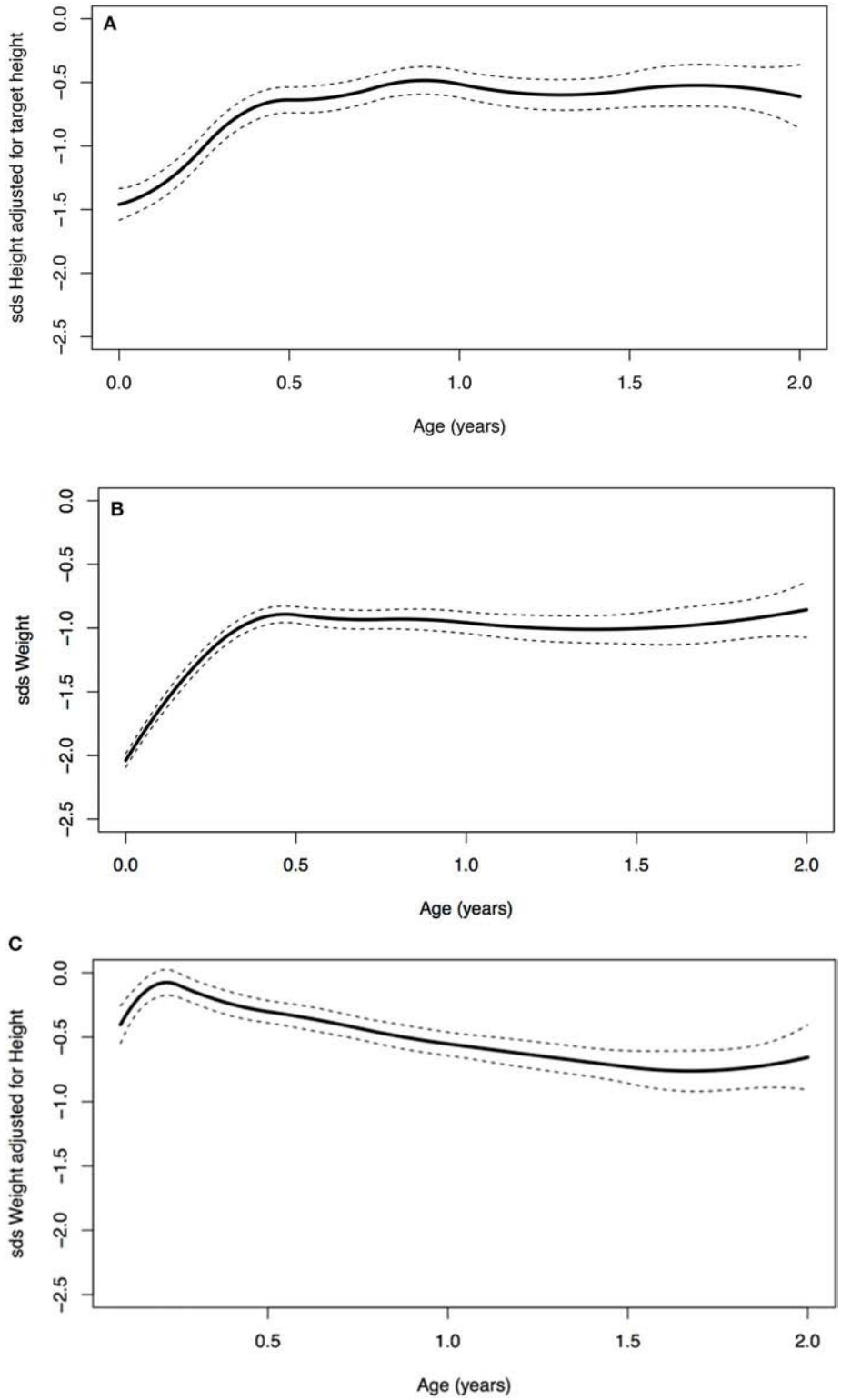

FIGURE 2 | (A) Age vs. standard deviation score (SDS) height adjusted for target height, **(B)** SDS weight, and **(C)** SDS weight for height.

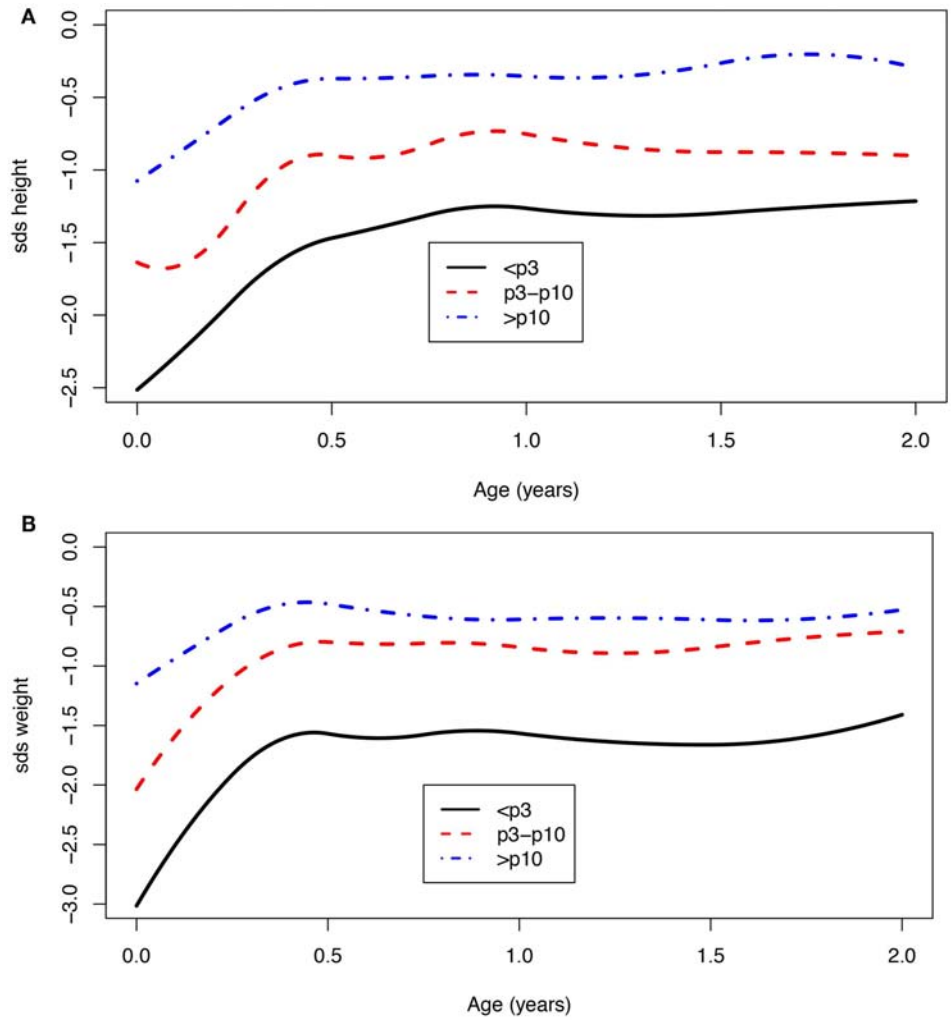

FIGURE 3 | (A,B) Age vs. SDS height and SDS weight for a birth weight below the 3rd, between 3rd and 10th, and above the 10th centile.

Respondents were slightly older, smoked less, higher educated, and more often Caucasian than non-responders. Baseline characteristics of the IoL group vs. EM are shown in **Table 2**. Babies born after EM were more severely growth restricted (birth with below the 3rd centile) than babies born after a policy of IoL (65.2 vs. 75.7%, $p < 0.05$).

The response rate was 53% ($n = 526$) (**Figure 1**). Of these patients, 292 were in the "randomized" group. For 487 children, at least 1 measurement of weight was available; for 458 children, 5 or more weight measurements were available; and for 71 children, 10 weight measurements were available. At 2 years of age, height measurement of 98 children and weight measurement of 100 children were available.

A significant increase in SDS in the first 2 years of life was seen (**Table 3**). At birth, the average SDS was −1.6 for height and −2.0 for weight in the entire group. At 2 years of age, the average SDS was −0.9 [mean increase from birth 0.7 (95% CI: 0.2; 1.0)] for height and −1.0 [mean increase from birth 1.0 (95% CI: 0.7; 1.2)] for weight (**Table 3** and **Figure 2**).

Children with a birth weight below the 10th centile ($<p10$) were born with a mean SDS of −1.8 for height ($n = 171$) and −2.4 for weight ($n = 382$), and at 2 years of age, the SDS was −1.1 for height ($n = 67$) and −1.2 for weight ($n = 69$). For children who were born with a birth weight that equals or was above the 10th centile ($>p10$), the mean SDS at birth was −1.1 for height ($n = 63$) and −1.2 for weight ($n = 141$), and at 2 years of age, −0.4 for height ($n = 30$) and −0.6 for weight ($n = 30$). Children born with a birth weight $<p10$ showed significantly more catch-up growth in weight at age 1 [mean difference −0.8 SDS (95% CI: −1.1; −0.5)] as well as at age 2 [mean difference −0.7 SDS (95% CI: −1.2; −0.2)] when compared to children born with a birth weight $>p10$ (**Figure 3**). The same effect was seen for children with a birth weight below the third centile (**Figure 3**). These children remain lower in SDS height and weight compared to the children born with a birth weight above the 3rd and 10th centiles.

Girls had lower weight SDS at birth [mean difference 0.3 SDS (95% CI: 0.1; 0.4)] and show more catch-up growth in weight

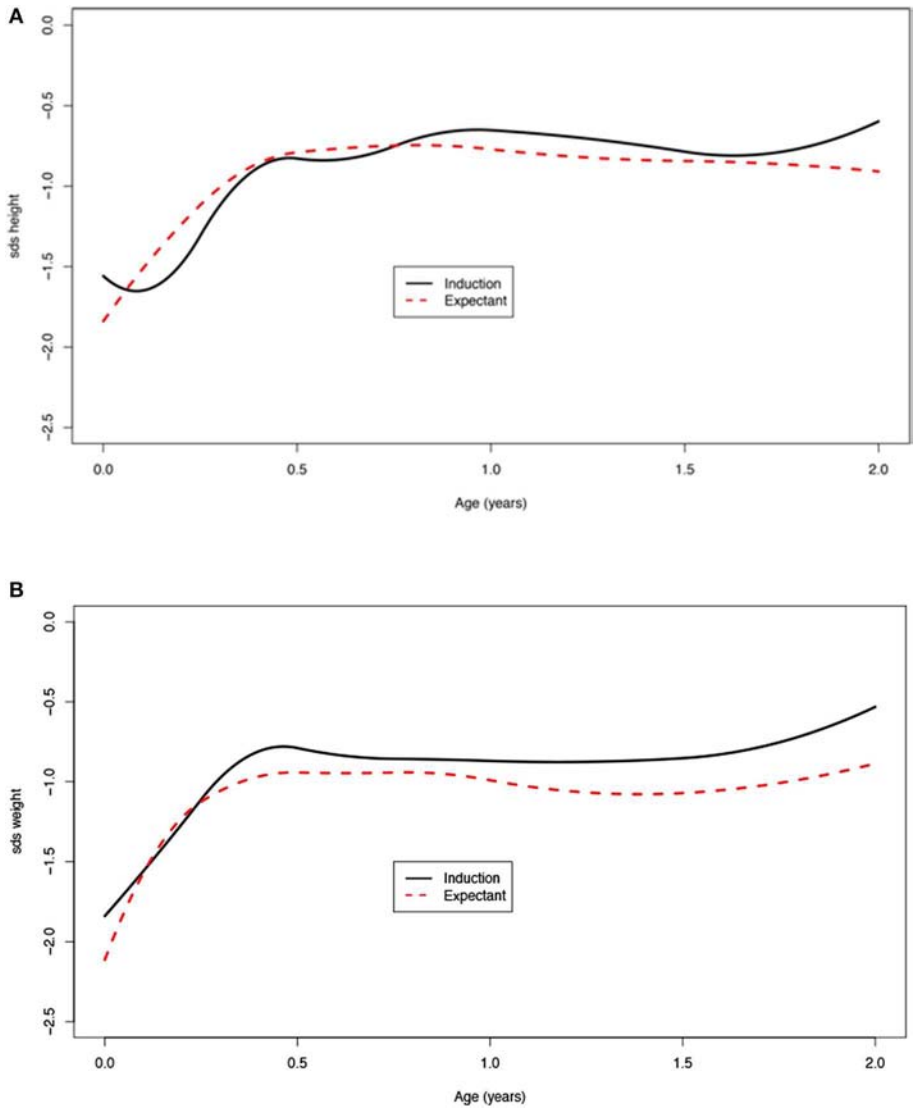

FIGURE 4 | (A,B) Age vs. SDS height and weight for induction of labor vs. expectant management.

particularly in the first month compared to boys (results in **Supplementary Material**).

Children born after obstetric policy of EM compared to children born after IoL showed significantly more catch-up growth in height [mean difference 0.7 (95% CI: 0.2; 1.3)] and weight [mean difference 0.5 (95% CI: 0.3; 0.7)] during the first month after birth. Thereafter, the growth patterns of the two groups were similar (**Figure 4**, **Table 4**).

Children with adverse neonatal outcome had lower SDS birth weight. We did not observe significantly more catch-up growth in this group, but numbers were low (**Supplementary Material**). Smoking during pregnancy resulted in 0.3 SDS (95% CI: −0.5; −0.1) lower birth weight in comparison to nonsmoking; a significant difference was seen in catch-up growth in the first 6 months after birth between children from smoking vs. nonsmoking mothers: after 6 months, the SDS scores were very similar in both groups (**Supplementary Material**). We did not see any effect of breastfeeding (**Supplementary Material**).

Complete catch-up growth in height was seen in 86% (n = 311) of children, and 84% (n = 335) complete catch-up in weight after 1 year. The mean target height SDS for the entire group was −0.3 SDS; the mean observed SD height score at 2 years of age was −0.9 SD, which is still significantly lower than the expected target height SD (p = 0.000).

When looking at neurodevelopmental and behavioral outcome problems at 2 years of age found by the ASQ and CBCL, we found that children with an abnormal score on the ASQ and were 6 and 12 months of age showed more catch-up in weight and were more below their target height when compared to the children with a normal ASQ (**Table 5**). We found no difference in catch-up height or weight in children with an adverse outcome for the CBCL (**Supplementary Material**). In a multivariate

TABLE 4 | Mean SDS height corrected for target height (TH), SDS weight, and catch-up growth (SDS weight–SDS birth) (± standard deviation) at different ages compared between children randomized for induction or expectant monitoring.

		Induction			Expectant management			Mean difference (95% CI)	*p*-value
		N	Mean	SD	*N*	Mean	SD		
SDS height-TH (SD)	1 month	97	−1.6	1.2	83	−1.2	1.1	−0.4 (−0.8;−0.1)	0.014
	6 months	112	−0.7	1.1	103	−0.5	1.2	−0.2 (−0.5; 0.1)	0.251
	9 months	92	−0.4	1.0	84	−0.3	0.9	−0.1 (−0.4; 0.2)	0.418
	12 months	78	−0.4	0.8	62	−0.4	1.3	0.1 (−0.3; 0.4)	0.759
	18 months	48	−0.5	0.8	51	−0.5	1.1	0.0 (−0.4; 0.4)	0.917
SDS birth weight	Birth	148	−1.8	0.7	121	−2.1	0.9	0.3 (0.1;0.5)	0.005
	1 month	115	−1.9	1.0	97	−1.7	1.0	−0.2 (−0.4; 0.1)	0.238
	3 months	125	−1.0	0.9	104	−1.1	0.9	0.1 (−0.2; 0.3)	0.638
	6 months	129	−0.9	1.0	112	−1.0	1.1	0.1 (−0.2; 0.4)	0.424
	9 months	105	−0.8	1.0	92	−0.9	1.0	0.1 (−0.2; 0.4)	0.600
	12 months	92	−0.9	0.9	66	−1.1	1.3	0.2 (−0.2; 0.5)	0.338
	18 months	57	−1.0	0.8	53	−1.0	0.9	0.0 (−0.3; 0.4)	0.879
Catch-up growth	1 month	115	0.1	0.9	97	−0.4	0.8	0.5 (0.3; 0.7)	0.000
SDS weight–SDS birth	3 months	125	−0.8	1.0	104	−1.0	0.9	0.2 (0.0; 0.5)	0.069
	6 months	129	−1.0	1.1	112	−1.1	1.1	0.2 (−0.1; 0.5)	0.205
	9 months	105	−1.0	1.2	92	−1.2	1.1	0.2 (−0.2; 0.5)	0.322
	12 months	92	−0.9	1.2	66	−1.0	1.1	0.1 (−0.3; 0.4)	0.667
	18 months	57	−0.9	0.8	53	−1.1	1.1	0.3 (−0.1; 0.6)	0.168

TABLE 5 | Mean SDS height corrected for target height (TH), SDS birth weight and catch up growth (SDS weight-SDS birth) (± standard deviation) at different ages compared between neurodevelopmental outcomes at age 2.

	Abnormal ASQ			
	Yes	No	Difference in mean (95% CI)	*p*-value
SDS height-TH (SD)				
6 months ($n = 376$)	−1.1 (1.1)	−0.6 (1.1)	−0.5 (−0.8; 0.2)	0.002
12 months ($n = 268$)	−1.1 (1.1)	−0.4 (1.0)	−0.7 (−1.0; −0.3)	0.002
18 months ($n = 355$)	−0.9 (1.2)	−0.5 (0.9)	−0.4 (−0.9; 0.2)	0.1
SDS Birth weight	−2.7 (0.8)	−1.9 (0.8)	−0.8 (−1.0; −0.6)	<0.001
Catch-up growth SDS weight-SDS birth				
6 months ($n = 443$)	1.3 (0.9)	1.0 (1.0)	0.3 (0.04; 0.6)	0.03
12 months ($n = 355$)	1.3 (0.9)	1.0 (1.1)	0.3 (0.005; 0.7)	0.047
18 months ($n = 188$)	1.2 (1.3)	0.9 (1.0)	0.3 (−0.2; 0.7)	0.3

logistic model, birth weight SDS had a strong association with an abnormal outcome of ASQ [a decrease of 1 birth weight SDS yielded a 2.6 (95% CI: 1.8; 3.5) times higher odds on an abnormal ASQ score]. Also, a moderate effect of gestational age [a 1-week longer gestational age increased the odds on an abnormal ASQ score by 1.21 (95% CI: 0.98; 1.50)] was found. Adding growth variables during the first month did not significantly improve the model and did not remarkably change these odds ratios.

DISCUSSION

Children born after EM of labor in FGR were more severely growth restricted at birth than children born after IoL (16), and these children exhibited more catch-up growth during the first month after birth. Also, growth-restricted children with a birth weight below the 10th centile showed more catch-up growth than those born with birth weights above the 10th centile. Catch-up growth occurs fastest in the first months after birth.

The fact that children born with a birth weight below the 10th centile showed significantly more catch-up growth than children born with a birth weight above the 10th centile indicates that more of these children were truly growth restricted and therefore might be at greater risk of developing metabolic syndrome in later life (7–9, 24). Children born after an EM also showed more catch-up growth during the first month after birth than children born after IoL. The difference in gestational age at birth between these children is on average 10 days. One could argue that keeping a fetus in an undernourished intrauterine environment for 10 days longer leads to more serious growth restriction and, in

turn, also to more catch-up growth. It is therefore likely that the antenatal decision either to actively end the undernourished status by inducing labor or to "wait and see"—an EM—influences long-term outcome and puts these children at a greater risk for metabolic and cardiovascular problems in later life. The long-term effects in relation to catch-up growth (i.e., during the first month, first years, etc.). remain unclear, and therefore, the long-term effects of catch-up growth during the first month after birth are undefined.

In children with neurodevelopmental problems at 2 years of age, more catch-up was seen in weight and height when compared to children with a normal neurodevelopmental outcome. This effect, however, disappears when correcting for weight SDS and gestational age at birth. We previously found that birth weight centile is the most important factor of influence on neurodevelopmental outcome at age 2, reflecting the dose effect of the severity of FGR (placental insufficiency) (17).

At 2 years of age, the majority of children of the DIGITAT cohort have height higher than 1.6 SD below their target height range and weight above −2 SDS, indicating that catch-up growth is, for the greatest part, complete (12). However, height and height SDS corrected for mid-parental height (target height SDS) and weight SDS are still significantly lower than expected in these children based on the norm population.

Our study is the first study to compare the effects of IoL with EM on postnatal catch-up growth in FGR. Previous studies have shown that children born after FGR exhibit catch-up growth in height and weight (1–3, 5, 6, 9, 12), but none of these studies studied catch-up growth in relation to obstetric policies to growth at 2 years of age. Another unique aspect of our study is that we included children based on suspected FGR and not based on actual birth weight, making comparisons with children with a birth weight above the 10th possible.

A weakness of our study is a possible response bias as data were obtained through postal inquiries and data were completed by the parents. We do, however, believe our measurements are reliable, as the children were measured by child health care professionals during routine scheduled child health care visits and parents were asked to provide us with those measurements. Respondents (mothers of the children) were slightly older, smoked less, higher educated, and more frequently Caucasian. Whereas, the percentage of growth-restricted children was comparable between non-respondents and respondents, there might have been more healthy life and feeding styles in respondent families; we do not know, however, to which direction our results would shift if the total sample would have been available. Unfortunately, our response rate was only around 50%, and the number of measurements at the age of 2 years is much lower. Within the group of respondents, we do not expect the group of children of whom we have measurements at age 2 to differ from the group of children without measurements at age 2. We suspect that missing values at 2 years are due to logistical reasons: the questionnaires were sent out around the age of 2 years (22–26 months of age) when a considerable group of parents had not yet been to the child health clinic for the scheduled 2-year visit and therefore did not have the measurements. We, therefore, do not expect a bias in our results, but it does influence our sample sizes.

The timing of IoL remains difficult however, as IoL before 38 weeks' gestation increases risks of complications due to late prematurity (25, 26). There are no known applicable biophysical markers that reflect maturation of fetuses; the best timing of induction depends, for the greatest part, on clinical assessment. We hypothesize that IoL in suspected FGR is optimal around 38 weeks (25), minimizing negative effects of being born late premature and hopefully preventing the risks from FGR and catch-up growth.

CONCLUSION

The majority of children born after FGR at term exhibit catch-up growth after birth and show complete catch-up growth after 2 years. After an EM policy, more children are severely growth restricted and show more catch-up growth during the first month after birth. A lower birth weight percentile was found as the most important factor influencing adverse neurodevelopmental outcome at age 2.

ETHICS STATEMENT

This study has been approved by the ethics committee of the Leiden University Medical Center (Ref. No. P04.210).

AUTHOR CONTRIBUTIONS

LW, SC, and KBo performed the analyses, drafted the initial manuscript, and approved the final manuscript as submitted. SS conceptualized and designed the study, interpreted the data, and critically reviewed the manuscript. JP, AW-L, HB, SG, FD, MP, KBl, FR, JL, and BM critically reviewed the manuscript. All authors have seen and approved the final manuscript.

FUNDING

The study was funded by ZonMw, the Netherlands Organization for Health Research and Development Health Care Efficiency Programme (grant number 945-04-558).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00274/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a past co-authorship with one of the authors SS.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 03 March 2020
doi: 10.3389/fendo.2020.00086

Is Umbilical Cord Blood Therapy an Effective Treatment for Early Lung Injury in Growth Restriction?

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Fetal growth restriction (FGR) and prematurity are often co-morbidities, and both are risk factors for lung disease. Despite advances in early delivery combined with supportive ventilation, rates of ventilation-induced lung injury (VILI) remain high. There are currently no protective treatments or interventions available that target lung morbidities associated with FGR preterm infants. Stem cell therapy, such as umbilical cord blood (UCB) cell administration, demonstrates an ability to attenuate inflammation and injury associated with VILI in preterm appropriately grown animals. However, no studies have looked at the effects of stem cell therapy in growth restricted newborns. We aimed to determine if UCB treatment could attenuate acute inflammation in the first 24 h of ventilation, comparing effects in lambs born preterm following FGR with those born preterm but appropriately grown (AG). Placental insufficiency (FGR) was induced by single umbilical artery ligation in twin-bearing ewes at 88 days gestation, with twins used as control (appropriately grown, AG). Lambs were delivered preterm at ~126 days gestation (term is 150 days) and randomized to either immediate euthanasia (unventilated controls, AG_{UVC} and FGR_{UVC}) or commenced on 24 h of gentle supportive ventilation (AG_V and FGR_V) with additional cohorts receiving UCB treatment at 1 h (AG_{CELLS}, FGR_{CELLS}). Lungs were collected at post-mortem for histological and biochemical examination. Ventilation caused lung injury in AG lambs, as indicated by decreased septal crests and elastin density, as well as increased inflammation. Lung injury in AG lambs was attenuated with UCB therapy. Ventilated FGR lambs also sustained lung injury, albeit with different indices compared to AG lambs; in FGR, ventilation reduced septal crest density, reduced alpha smooth muscle actin density and reduced cell proliferation. UCB treatment in ventilated FGR lambs further decreased septal crest density and increased collagen deposition, however, it increased angiogenesis as evidenced by increased vascular endothelial growth factor (VEGF) expression and vessel density. This is the first time that a cell therapy has been investigated in the lungs of growth restricted animals. We show that the uterine environment can alter the response to both secondary stress (ventilation) and therapy (UCB). This study highlights the need for further research on the potential impact of novel therapies on a growth restricted offspring.

Keywords: growth restriction, ventilation induced lung injury, umbilical cord blood (UCB), treatment, animal model

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Edited by:
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Specialty section:
This article was submitted to Reproduction, a section of the journal Frontiers in Endocrinology

Received: *15 November 2018*
Accepted: *11 February 2020*
Published: *03 March 2020*

Citation:
Allison BJ, Youn H, Malhotra A, McDonald CA, Castillo-Melendez M, Pham Y, Sutherland AE, Jenkin G, Polglase GR and Miller SL (2020) Is Umbilical Cord Blood Therapy an Effective Treatment for Early Lung Injury in Growth Restriction? Front. Endocrinol. 11:86. doi: 10.3389/fendo.2020.00086

INTRODUCTION

Fetal growth restriction (FGR) is a common complication of pregnancy, where a fetus fails to reach its expected growth potential, primarily due to placental insufficiency (1). FGR significantly increases the risk of morbidity and pulmonary conditions following preterm birth, with increased rates of bronchopulmonary dysplasia and pulmonary hypertension (2, 3). Despite the increased risk of pulmonary complications, lung pathology following FGR remains contentious. We and others have found comparable lung weight, structure, surfactant protein expression, and ventilation requirements compared to appropriately grown (AG) cohorts (4, 5). However, it is evident that early and late onset FGR have differential effects (6), and animal studies to date have primarily induced FGR during late gestation, and it is, thus, possible that crucial lung development has already occurred at this stage (7); whereas preclinical studies of long term growth restriction describe altered surfactant protein (8, 9) disrupted alveolarization, with thickened parenchyma (10) and large alveoli resulting in reduced alveolar and vascular density (11).

There is currently no cure or therapy for FGR. Current treatment of FGR primarily involves the adjustment of the delivery time, thus infants are often delivered preterm (<37 weeks gestation), particularly those with early-onset FGR (12). Prematurity itself is a significant risk factor for pulmonary morbidity and necessitates medical interventions such as mechanical ventilation. Whilst ventilation is usually essential for survival in such scenarios, it has the potential to exacerbate pathology in FGR lungs, particularly since lung development may already be adversely affected by the chronic hypoxemia caused by placental insufficiency (11). The resultant lung injury after birth is known as ventilation induced lung injury (VILI). VILI and elevated inflammation cause direct tissue injury and in turn, exacerbate lung inflammation. Long term ventilation can reduce alveoli number, disrupt vasculature and alveolar architecture (13–16), hampering lung mechanics and necessitating the further need for assisted ventilation. Current treatment focuses on ensuring the survival of the FGR infant while ameliorating the detrimental effects of FGR on the lungs.

Umbilical cord blood (UCB) cells have been highlighted as a potential treatment for infants born preterm, due to their potent anti-inflammatory properties and easy access (17). Preclinical studies using specific stem like cell populations present within UCB have shown promising anti-inflammatory and immune modulatory effects in VILI of preterm animals (15, 18). UCB provides a unique source of the functionally important stem like cells that may each play a modulatory role in preventing injury (19). UCB therapy has been examined for prevention and repair of brain injury (20, 21), and has also shown promise in clinical trials where administration improved motor and neurodevelopmental outcomes in children with cerebral palsy (22, 23). UCB is comprised of many cell types including cells that mediate hematopoiesis and vascular growth (24–27). UCB also show strong anti-inflammatory benefits, and they are a feasible postnatal treatment with low immunogenicity (25, 26, 28). Within the lung, UCB therapy is thought to reduce inflammation through paracrine effects. Accordingly, we used our established model of ovine fetal growth restriction to examine (i) the effects of preterm birth and ventilation on FGR lungs, and (ii) if UCB could be a potential new treatment for VILI in FGR and/or AG infants. To focus on acute inflammation and injury, this study examined the first 24 h postnatally in FGR and AG preterm lambs.

METHODOLOGY

Umbilical Cord Blood Collection

Umbilical cord blood (UCB) was collected from separate healthy term ovine pregnancies. UCB was collected during cesarean section under general anesthesia. Approximately 90 mL of UCB was collected from the umbilical veins into heparinized tubes. UCB was diluted 1:1 with phosphate buffered saline and centrifuged at 3,200 rpm at RT for 12 min with no brake. The buffy coat was isolated to obtain the mononuclear cells (MNCs) and red blood cell lysis of this fraction performed. Cells were counted using trypan blue exclusion and a hemocytometer, and cells were cryopreserved at ∼25 million cells/ml in freeze media (10% DMSO, 40% FBS and 50% DMEM/F12) until required. A minimum of three cryopreserved UCB donors was pooled after thawing and before administration to reduce intra-sample UCB variation.

Fetal Surgery

Aseptic surgery was performed on anesthetized (sodium thiopentone 20 mL; Pentothal; Boehringer Ingelheim, Australia; maintenance inhaled isoflurane 2–5%) Border-Leicester pregnant ewes ($n = 17$) carrying twin-pregnancies at 88 days gestation (term is 150 days). Prophylactic antibiotics were administered via the maternal jugular vein, including 5 mL of Engemycin (Engemycin 100, Coopers, MSD Animal Health, New Zealand) and 1 g of ampicillin (Ampicyn 1 g, Mylan N.V., USA). Following a thorough cleaning of the surgical sites, the fetus was exposed via cesarean section. Marcain (5 mL, Marcain (0.5%) with Adrenaline, Aspen Pharmacare Australia NSW, Australia) was applied to all surgical sites prior to incision to provide analgesic coverage. Single umbilical artery ligation was performed by placing two silk ties tightly around one of the umbilical arteries, that causes chronic placental insufficiency and fetal growth restriction (FGR). In control twins, the umbilical cord was handled but not ligated. The fetus was returned to the uterus and abdominal incisions were repaired. A maternal jugular vein catheter was inserted for antibiotic administration. Following surgery ewes were randomly allocated to an experimental group (UVC $n = 6$ ewes, V $n = 5$ ewes or CELLS $n = 6$ ewes).

For 3 consecutive days after surgery, antibiotics [to the fetus (Ampicillin, 1 g via the amniotic catheter] and the ewe [Engemycin 5 mL intravenous (i.v.)] and analgesia

[Panadol (100 mg/mL, Apotex, NSW, Australia) suppository] were administered.

Experimental Design

The ewe and fetuses were monitored daily until 124 days of fetal gestation. At 124 and 125 days, ewes received 11.4 mg betamethasone intramuscularly (Celestone Chronodose, Schering Plow, Sydney, Australia). At 126 days, ewes ($n = 11$) and their fetuses ($n = 21$) in the ventilation groups (AG_V $n = 6$, FGR_V $n = 6$ and AG_{CELLS} $n = 6$, FGR_{CELLS}, $n = 5$) underwent an additional cesarean section or post-mortem (AG_{UVC} $n = 6$, FGR_{UVC} $n = 5$). At this time, there had been $n = 2$ *in utero* deaths in the FGR groups; $n = 1$ in the FGR_{UVC} group and $n = 1$ in the FGR_{CELLS} group, hence the reduced number in these two groups at this timepoint. Following maternal anesthesia (sodium thiopentone 20 mL; maintenance inhaled isoflurane 2–5%), the first lamb was exteriorized and intubated with an endotracheal tube (size 4.0 mm). Lung liquid was drained passively and a transcutaneous arterial oxygen saturation (SpO_2) probe (Masimo, Radical 4, CA, USA) was placed around the right forelimb of the lamb and the output digitally recorded.

The umbilical cord was then clamped and cut, the lambs were delivered, dried, weighed and placed on an infant warmer (Fisher and Paykel Healthcare, Auckland, New Zealand) for initiation of assisted ventilation. An umbilical vein and artery were immediately catheterized for maintenance of anesthesia and analgesia (Alfaxane i.v. 5 mg/kg/h; Jurox, East Tamaki, Auckland, New Zealand). Arterial pressure was digitally recorded in real-time (1 kHz, Powerlab; ADInstruments, Castle Hill, NSW, Australia). The lambs were anesthetized for the entirety of the experiment to prevent spontaneous breathing. Ventilation was commenced using positive pressure ventilation with PIP set at 30 cmH_2O and PEEP at 5 cmH_2O (Babylog 8000+, Dräger, Lübeck, Germany): inspiratory time was 0.4 s and expiratory time was 0.6 s. Lambs were ventilated with warmed, humidified gas with an initial fraction of inspired oxygen (F_iO_2) of 0.4 and subsequently adjusted to maintain SaO_2 between 90 and 95%. At 10 min, all lambs received surfactant (Curosurf, 100 mg/kg, Chiesi Farmaceutica, Italy). At 20 min, ventilation continued in volume guarantee mode set at 5–7 ml/kg, which is the tidal volume for lambs at this gestation (29). Physiological parameters pH and $PaCO_2$ were kept within normal limits (7.2–7.4 and 45–55 mmHg, respectively) by adjusting the ventilator rate and inspired O_2 levels. Lamb well-being was monitored throughout ventilation via assessment of the partial pressure of arterial oxygen (PaO_2) and carbon dioxide ($PaCO_2$), oxygen saturation (SaO_2), pH, hematocrit, glucose and lacate with regular blood gas samples (ABL 700 blood gas analyzer; Radiometer, Copenhagen, Denmark). Lambs were ventilated for 24 h.

For groups that received UCB (AG_{CELLS} $n = 6$ and FGR_{CELLS} $n = 5$), 25 million cells/kg was administered intravenously to lambs at 1 h after birth, control groups (AG_V $n = 5$ and FGR_V $n = 5$) were administered the equivalent volume of saline. UCB cells were quantified via cell counts of UCB mononuclear cells to establish an accurate dose prior to administration.

Post-mortem

At 24 h, ventilated lambs were euthanized with an overdose of 20 mL of phenobarbitone, whilst unventilated control groups were immediately euthanized at 125 ± 1 days gestation via an overdose of phenobarbitone. Lambs were weighed and lungs isolated for collection. The left bronchus was ligated before the left lung was removed distal to the ligature. The left lung was snap frozen in liquid nitrogen for RNA processing. The right whole lung was pressure fixed at 20 cmH_2O via the trachea with formalin. Nine sections (2 cm^3) of the lung were randomly selected from an area devoid of major airways from each lobe (upper, middle, lower) and processed for assessment of lung histology. Lung sections were embedded in paraffin, then cut into 5 μm sections and mounted on to Superfrost Plus slides for histological and immunohistochemical analysis.

Detecting Stem Cell Migration

To detect if UCB stem cells were present in the lungs of treated lambs, the UCB cells were tagged with carboxyfluorescein succinimidyl ester (CFSE) before administration (30). Cut lung sections were dewaxed and counter-stained in Hoechst (Invitrogen, USA) and coverslipped. Stem cell identification was conducted using fluorescent microscopy (Olympus BX-41, Japan).

Histological Analysis of Lung Morphology

Gross histological pathology and parenchymal elastin was detected via Hematoxylin and Eosin and Hart's elastin stains, respectively, as previously described (31) Masson's Trichrome was used to identify collagen fibers (32). Three sections (obtained as described above) were randomly selected for each histological assessment. Quantification of histology is outlined below.

Immunohistochemistry

Lung tissue was immunostained for Ki67, α-smooth muscle actin and CD45. Ki67 and α-smooth muscle actin immunostaining was carried out as previously described [(5, 33) see **Supplementary Table 1**]. For immunostaining of CD45, slides were heated in a 60°C oven for 2 h to remove excess wax, followed with histolene clearing and ethanol rehydrating steps. Antigen retrieval was performed by boiling tissue sections in 0.01 M Citrate buffer (pH 6.0) for 3 × 10 min bursts. Sections were then washed in phosphate buffer solution (PBS) before endogenous peroxidase in the tissue was blocked with 3% hydrogen peroxide for 10 min. Tissue sections were washed and then slides were blocked in Serum-Free Protein Block (DAKO) before incubation with the primary antibody CD45 (BD Pharmingen Rat Anti-Mouse, 1:500) for 60 min. Sections were washed in PBS and then incubated with biotinylated secondary antibody (Rabbit anti-mouse, 1:200) for 60 min followed with streptavidin horseradish peroxidase and developed with diaminobenzidine (DAB) and hydrogen peroxide. Sections were counterstained with hematoxylin and dehydrated with ethanol and histolene before mounting with a coverslip. All immunostains were performed in the presence of a negative control.

TABLE 1 | Total sample size, animal and lung weights and sex of fetuses and lambs.

	AG_{UVC}	FGR_{UVC}	AG_V	FGR_V	AG_{CELLS}	FGR_{CELLS}
n	6	5	6	6	6	5
Animal weight (kg)	3.56 ± 0.13	2.25 ± 0.17#	3.21 ± 0.17	2.46 ± 0.22#	3.40 ± 0.18	2.29 ± 0.17#
Lung corrected for body weight (g/kg)	35.94 ± 1.98	31.04 ± 3.07	29.33 ± 0.91	30.36 ± 3.22	27.32 ± 1.55	29.44 ± 1.90
Males, n (%)	5 (83)	2 (40)	2 (33.3)	2 (33.3)	5 (83)	2 (40)

AG, appropriately grown; UVC, unventilated control; FGR, growth restricted; V, ventilated; CELLS, animals treated with umbilical cord blood cells. #Indicates $p < 0.05$ for growth effects using a 2-way ANOVA.

Cytokine Array

To assess cytokine expression in the lungs, frozen lung tissue was weighed out in 50–100 mg quantities, for protein expression of pro-inflammatory and anti-inflammatory cytokines. The concentrations of *interferon gamma (IFNγ)*, *interleukin (IL)-17A, IL-21, IL-8, IL-10 TNFα*, and *VEGF-A* in lung tissue lysate were measured using an ovine cytokine array (ovine QAO-CYT-1-1, RayBiotech, Georgia, USA).

Analysis

For histological and immunohistochemistry analysis, five random fields of view were taken of each section and analyzed by a single blinded observer (H.Y.). Images were non-overlapping and excluded large airways or vessels.

Lung morphology was assessed through quantification of tissue to airspace ratio and density of secondary septal crests as previously described (4). Elastin, collagen and αSMA density were assessed through Smart Segmentation on Image Pro Premier (Media Cybernetics, USA) (16). Elastin and collagen were then expressed as ratios of lung tissue. Manual point counting was utilized to assess Ki67 and CD45 to tissue ratios (16). Measurement of vascular vessel number was assessed using αSMA immunostained tissue by a single observer blinded to the experimental group (B.J.A.). Vessels were identified by positive staining and were only included when a full cross section of the vessel was visible in the field of view.

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed with SPSS using a mixed model using growth and treatment as factors in all immunohistochemical and morphological assessments and growth, treatment and time in ventilation parameters. Where significant interactions were detected, differences were isolated with *post-hoc* Tukey's testing. Statistical significance was accepted as $P < 0.05$.

RESULTS

Lamb Characteristics

Lamb characteristics are presented in **Table 1**. Single umbilical artery ligation (SUAL) resulted in ∼30% overall reduction in birth weight in FGR lambs. SUAL also resulted in the death of two fetuses, one in the UVC and one in the CELLS group. FGR_{UVC} weighed 37% $<AG_{UVC}$ ($P = 0.0001$), whilst FGR_V weights were 23% lower than AG_V ($P = 0.04$) and FGR_{CELLS} lambs weighed 32% $<AG_{CELLS}$ ($P = 0.01$, **Table 1**). Lung weight, corrected for body weight, was not different across groups.

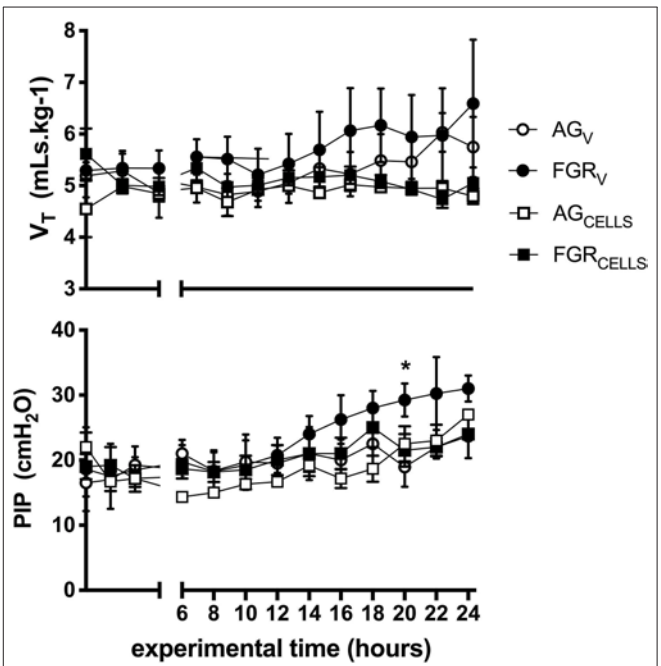

FIGURE 1 | Ventilation parameters. Mean ± SEM tidal volume V_T ($mLs.kg^{-1}$) and peak inspiratory pressure (PIP) in appropriately grown (AG_V, white circles, $n = 5$), growth restricted (FGR_V, black circles $n = 5$) and appropriately grown and growth restricted treated with umbilical cord blood cells (AG_{CELLS}, white squares $n = 6$; FGR_{CELLS}, black squares $n = 5$) over the experimental period (hours) *indicates significant differences ($p < 0.05$) across time.

Ventilation Parameters

There were no differences in tidal volumes (V_T, 5–6 mL/kg) between groups. Peak inspiratory pressure (PIP) required to achieve V_T was initially not different between groups, however PIPs were significantly ($P > 0.003$) increased at 20 h in FGR_V lambs compared to all groups (**Figure 1**). There was no effect of FGR or UCB on the requirement for PIP. Lung compliance was not different between groups (data not shown).

Stem Cell Migration

Lung tissue was examined for the presence of CFSE tagged UCB cells in all ventilated groups (**Figure 2**). Fluorescing cells were

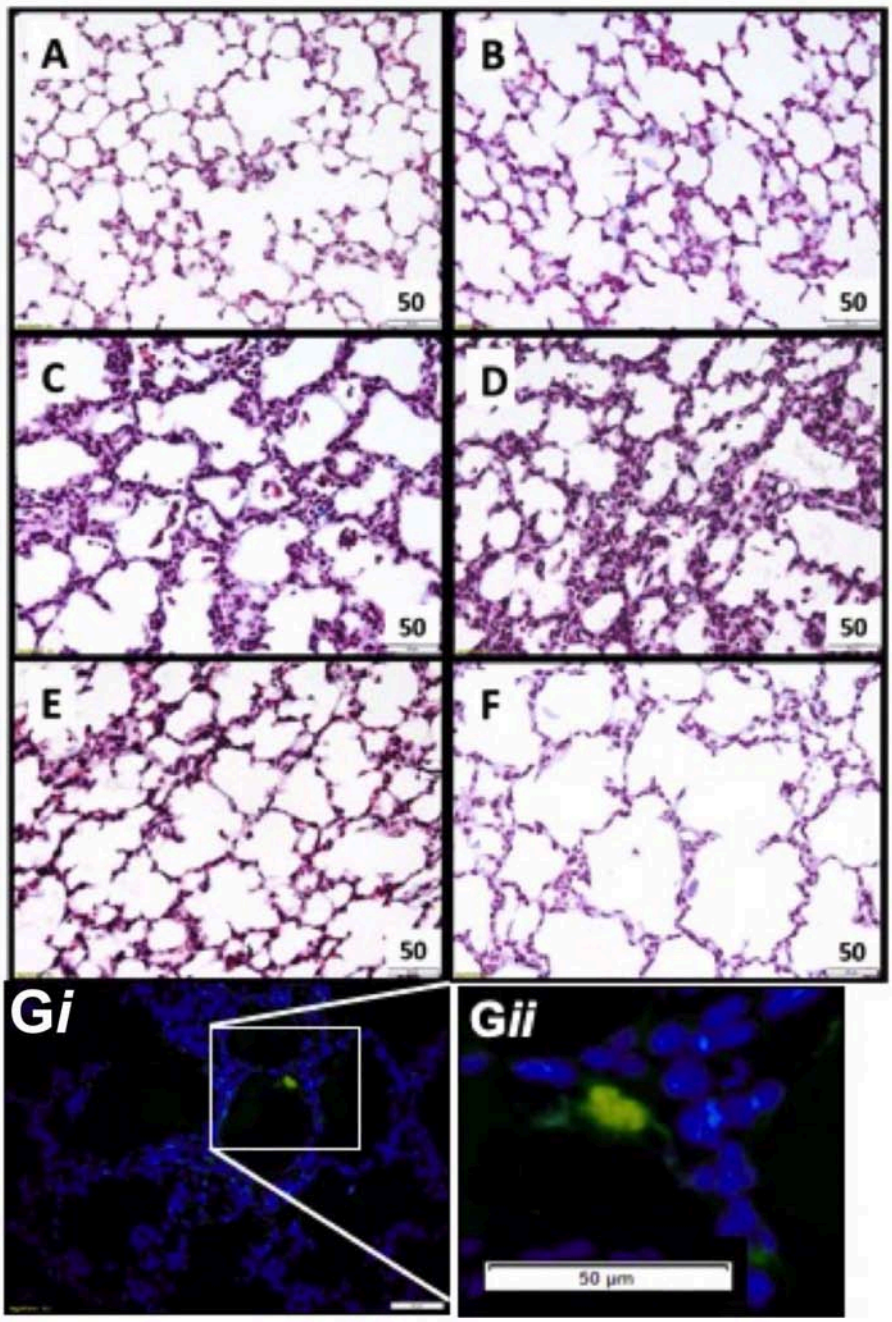

FIGURE 2 | Representative lung morphology images. Photomicrographs of Masson Trichrome stained sections in appropriately grown (**A** AG_{UVC}, **C** AG_{VENT}, **E** AG_{CELLS}) and growth restricted (**B** FGR_{UVC}, **D** FGR_{VENT}, **F** FGR_{CELLS}) animals. Fluorescent tagged cell in lung parenchyma UCB cells present within parenchymal lung tissue (blue) and a fluorescing UCB cell (green). Magnification ×400 (**G***i* and at higher magnification **G***ii*).

apparent in all cell treated animals, and were not present in saline controls (**Figure 2G**).

Lung Morphology

Two lungs were not appropriately fixed (one from FGR_{UVC} and one from FGR_{CELLS}) and were thus excluded from morphological and immunohistochemical analysis. Final sample size for morphological and immunohistochemical analysis is AG_{UVC} $n = 6$, AG_V $n = 6$, AG_{CELLS} $n = 6$, FGR_{UVC} $n = 4$, FGR_V $n = 6$ and FGR_{CELLS} $n = 4$.

Tissue to airspace ratio was not altered by FGR or ventilation (**Figure 3A**). Heterogeneous lung injury was observed in AG_V and FGR_V compared to their unventilated cohorts, with areas containing thickened blood-air barriers, contrasting against

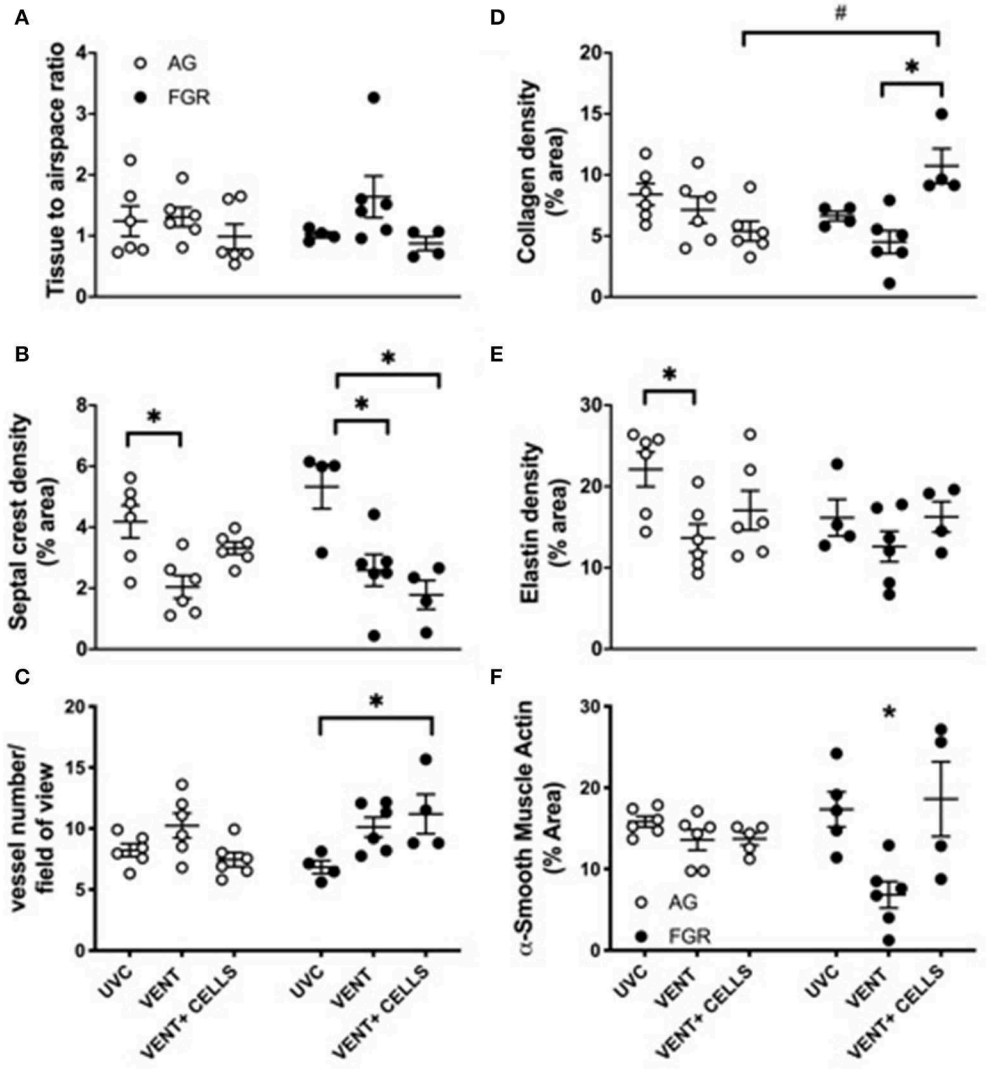

FIGURE 3 | Lung parenchymal and vascular structure. Data are mean ± SEM tissue airspace ratio **(A)**, secondary crest density **(B)**, arteriolar vessel wall number **(C)** and collagen **(D)** elastin **(E)** and α-smooth muscle actin **(F)** density (corrected for total tissue area) in appropriately grown (AG) and growth restricted (FGR) unventilated controls (AG_{UVC} and FGR_{UVC}, white circles), following ventilation (AG_{VENT} and FGR_{VENT}, black circles) and following ventilation and cell treatment (AG_{CELLS} and FGR_{CELLS}, gray circles). Data were compared using a two-way ANOVA. *Indicates $p < 0.05$ treatment effects and #indicates $p < 0.05$ growth effects using a two-way ANOVA.

other regions showing prominent airway enlargement. This heterogeneity was reduced with UCB (AG_{CELLS} and FGR_{CELLS}), although alveoli remained enlarged (**Figure 2**). Septal crest density was significantly reduced following ventilation compared to unventilated controls (**Figure 3B**) resulting in a 57.6% reduction in AG lambs (AG_{UVC} 4.2 ± 0.5 vs. AG_V 2.0 ± 0.4, $P = 0.0001$) and 44.6% reduction in FGR lambs (FGR_{UVC} 5.3 ± 0.7 vs. FGR_V 2.5 ± 0.5, $P = 0.002$, **Figure 3B**). Treatment with UCB restored septal crest density in AG (AG_{CELLS} 3.3 ± 0.2, $P = 0.02$) but not FGR lambs.

Vessel number, as assessed in α-smooth muscle actin-stained lungs (**Figure 3C**). was significantly increased in ventilated growth restricted lambs treated with UCB compared to unventilated, growth restricted lambs (FGR_{CELLS} 11.2 ± 1.6 vs. FGR_{UVC} 6.8 ± 0.5, $P = 0.04$). No differences in vessel density were observed across groups in the AG lambs.

Collagen density was not altered in either AG or FGR lambs following ventilation (**Figure 3D**). Treatment with UCB significantly increased collagen density in FGR_{CELLS} animals compared to FGR_V and AG_{CELLS} lambs (FGR_{CELLS} 10.7 ± 1.4% vs. AG_{CELLS} 5.3 ± 0.8% and FGR_V 4.5 ± 0.9%; $p > 0.02$).

Elastin density was significantly reduced in ventilated AG lambs compared to AG control lambs (AG_{UVC} 22.1 ± 2.2% vs. AG_V 13.6 ± 1.7%, $P < 0.05$). Elastin density was restored following treatment with UCB in AG lambs (**Figure 3E**). Elastin density was not different in FGR lambs either with ventilation or UCB treatment.

We assessed positively stained α-smooth muscle actin tissue to determine density (**Figure 3F**). Ventilation of FGR lambs

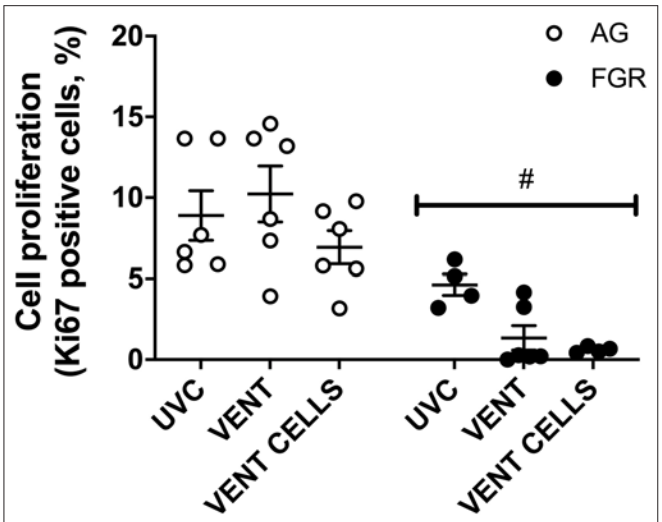

FIGURE 4 | Cell proliferation. Data are mean ± SEM Ki67 (cell proliferation marker) positive cells in appropriately grown (AG, white) and growth restricted (FGR, black) unventilated controls (AG_{UVC} and FGR_{UVC}), following ventilation (AG_{VENT} and FGR_{VENT}) and following ventilation and cell treatment (AG_{CELLS} and FGR_{CELLS}). Data were compared using a two-way ANOVA. # Indicates $p < 0.05$ treatment effects using a two-way ANOVA.

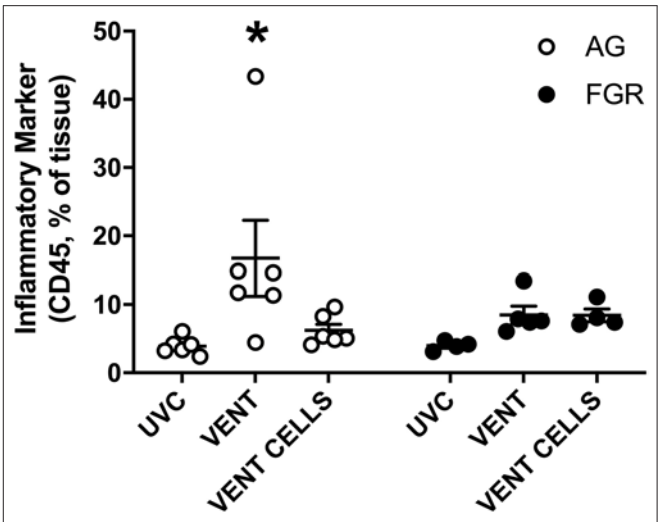

FIGURE 5 | Inflammation. Data are mean ± SEM CD45 (inflammation marker) positive cells in appropriately grown (AG, white) and growth restricted (FGR, black) unventilated controls (AG_{UVC} and FGR_{UVC}), following ventilation (AG_{VENT} and FGR_{VENT}) and following ventilation and cell treatment (AG_{CELLS} and FGR_{CELLS}). Data were compared using a two-way ANOVA. *Indicates $p < 0.05$ treatment effects using a two-way ANOVA.

significantly reduced α-smooth muscle actin density (FGR_V 6.8 ± 1.3% vs. FGR_{UVC} 17.3 ± 2.1%). Treatment with UCB reversed this finding (FGR_V 6.8 ± 1.3% vs. FGR_{CELLS} 18.6 ± 4.6%). α-smooth muscle actin density was not different in AG lambs either with ventilation or UCB treatment.

Cell Proliferation

Cell proliferation was assessed in lung parenchyma using the proliferation marker Ki67. Cell proliferation was significantly decreased ($P < 0.0001$, **Figure 4**) in FGR, compared to AG groups. Cell proliferation was significantly reduced by 80% in FGR_{VENT} compared to AG_{VENT}, and also decreased in FGR_{CELLS} compared to AG_{CELLS} (by 90%).

Inflammation

We used immunohistochemical analysis of CD45 to visually examine the infiltration of inflammatory cells into pulmonary tissue. Ventilation induced an inflammatory response in AG lambs (AG_{UVC} 3.9 ±.5 cells vs. AG_V 16.7 ± 5.5 cells, $P = 0.0079$). Inflammatory cell infiltration of the lungs was attenuated by treatment with UCB (**Figure 5**, $p < 0.05$) in AG lambs. Neither ventilation nor cell treatment induced a significant inflammatory response within the lungs of FGR lambs (FGR_{UVC} vs. FGR_V $p = 0.5$; FGR_V vs. FGR_{CELLS} $p = 0.9$).

A cytokine array was performed to further characterize the inflammatory profile within the lungs. Pro-inflammatory marker IL-8 was significantly increased in response to ventilation, in both AG and FGR lambs ($P < 0.05$), while treatment with UCB did not reduce IL-8 levels (**Figure 6A**). UCB treatment significantly increased IL-21 levels in FGR and AG lambs (**Figure 6E**). VEGF concentration was significantly increased in FGR lambs treated with UCB compared to both FGR_{UVC} ($P = 0.007$, **Figure 6E**) and FGR_V ($P = 0.04$) lambs. However, treatment with UCB cells significantly increase VEGF protein levels in FGR lambs compared to AG lambs treated with UCB cells (FGR_{CELLS} 16.6 ± 0.7 vs. AG_{CELLS} 9.9 ± 1.8, $P = 0.002$). There was no difference in TNFα, IL-17A, IL-10 or IFNγ levels between groups (**Figures 6B–D,F**, respectively).

DISCUSSION

Postnatally, FGR infants have increased risk of lung injury and bronchopulmonary dysplasia (BPD). Stem cell therapy has proven benefits to reduce VILI in preterm infants (34) as well as in reducing BPD incidence in preterm humans (33) and in animal models of neonatal lung injury (35). However, no previous studies have investigated if stem cell therapy is also beneficial for very low birthweight infants affected by growth restriction. In the current model, UCB therapy attenuated injury in AG but not FGR lambs. Our findings in appropriately grown lambs confirm previous studies showing improved lung structure following administration of placental stem-like cells, such as human amnion epithelial cells (34). Therefore, our current study increases the body of evidence for the use of UCB as an effective therapy for VILI in preterm infants who are appropriately grown. UCB treatment in FGR lambs increased pulmonary vascularization, but did not improve structural deficits in secondary septal crests, and increased collagen deposition, which is an early marker of fibrosis. Our research demonstrates that after 24 h of ventilation, UCB therapy shows differential effects in appropriately grown and growth restricted lambs, where protection from VILI was evident in AG lambs but not FGR lambs.

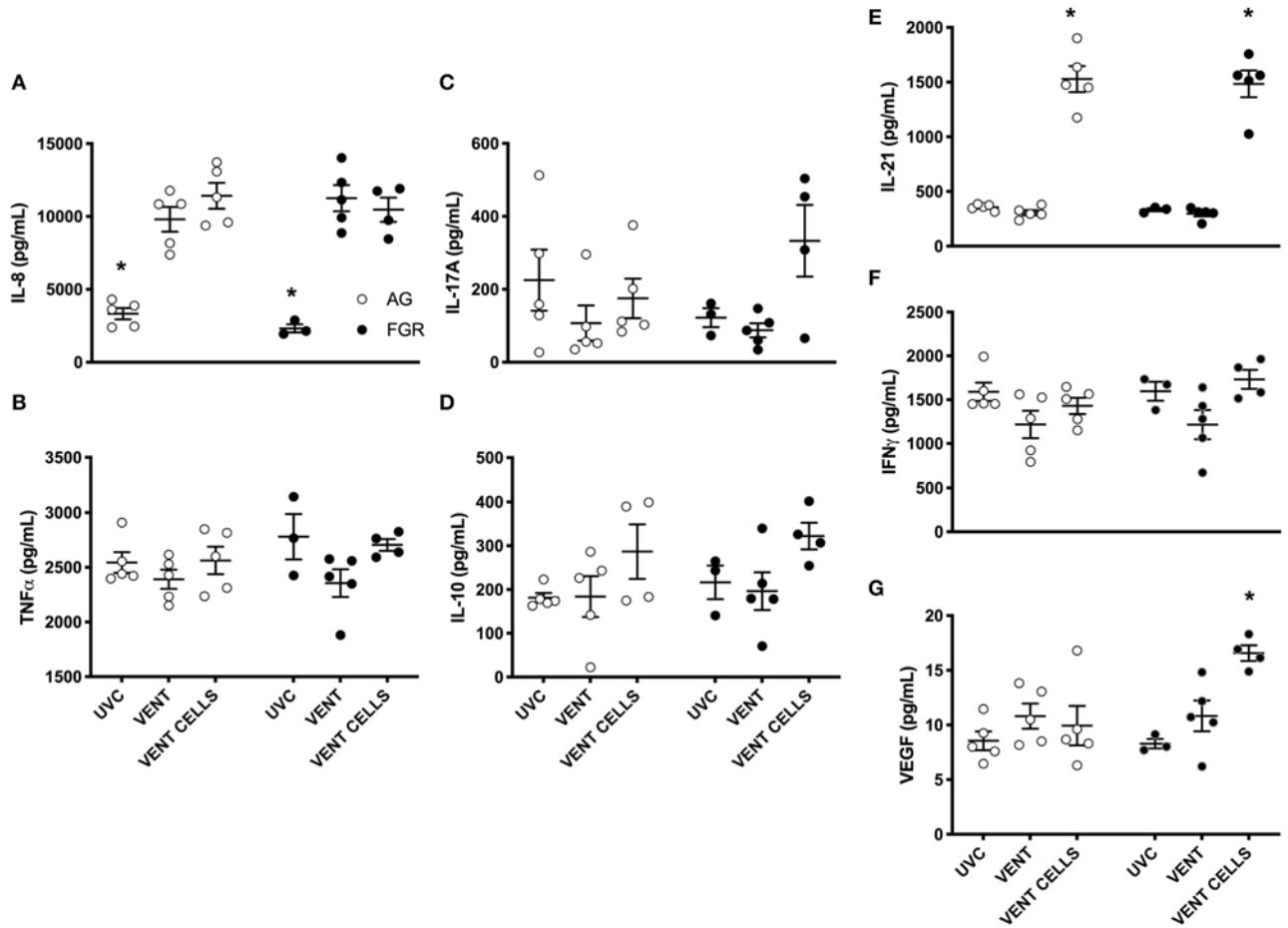

FIGURE 6 | Inflammatory proteins. Data are mean ± SEM interleukin *(IL)-8* **(A)**, tumor necrosis factor alpha *(TNFα)* **(B)** , *IL-17A* **(C)**, *IL-10* **(D)**, *IL-21* **(E)** interferon gamma *(IFN)* **(F)**, and vascular endothelial growth factor *(VEGF)* **(G)**, cytokine levels in appropriately grown (AG, white) and growth restricted (FGR, black) unventilated controls (AG_{UVC} and FGR_{UVC}), following ventilation (AG_{VENT} and FGR_{VENT}) and following ventilation and cell treatment (AG_{CELLS} and FGR_{CELLS}). *Indicates $p < 0.05$ treatment effects using a two-way ANOVA.

In the current study, we found little difference in the baseline lung morphology between the preterm unventilated FGR and AG fetuses, in line with previous observations from our group (4), although we induced early-onset placental insufficiency and FGR in this study, where we have previously examined late-onset (36). We hypothesized that longer exposure to placental insufficiency over a period of critical lung development would lead to the arrest of alveolar development as observed in other preclinical FGR studies (10, 11, 37). The latter being a probable mechanism for the increased risk of BPD (3) in this cohort. However, we did not see detectable differences in lung weights, when corrected for body weight, or baseline lung morphology in this study. Differences between the mode of inducing FGR and timing of compromise are most likely to contribute to differences in experimental outcomes. It is interesting to note that, despite a lack of gross or microscopic changes in lung morphology before ventilation, critical differences in response to ventilation were evident in the current study between AG and FGR lambs, suggesting subclinical alterations in lung development and/or biochemistry.

We have previously shown that AG and FGR newborns do not have significantly different ventilator requirements in the first 2 h of life (4, 38), however, in this study we extended these findings to show that, with a prolonged period of ventilation, FGR lambs begin to require a greater PIP to maintain V_T, suggesting stiffer and less compliant lungs, a change that was subclinical throughout our experiment period as shown by dynamic compliance. All our lambs received antenatal betamethasone, which enhances surfactant production (39) and pulmonary function (40), and temporarily preserves lung compliance (41, 42) however, this may have had decreased efficacy in FGR. The rise in PIP over time may be a precursor to worsening VILI, lung compliance and ventilatory requirements, and certainly suggests that a longer period (>24 h) of study is necessary to tease apart differences associated with FGR.

Mechanical trauma as occurs with assisted ventilation induces an acute inflammatory response that initiates the inflammatory cascade and stimulation of inflammatory cytokine production (14, 16, 35). This was confirmed in the current study with ventilation significantly increasing pro-inflammatory cytokine IL-8 in FGR and AG lambs. In keeping with pulmonary IL-8 upregulation, infiltration of immune cells into the lungs (as evident by $CD45^+$ expression) was also increased with ventilation, albeit this was statistically significant only in the AG lambs. Upregulation of inflammation following lung injury is well-described (4, 14, 16, 43, 44). Interestingly, IFNγ and TNFα were not altered in ventilated FGR or AG lambs. IFNγ is recognized as a key pro-inflammatory cytokine that has previously been shown to be up-regulated in response to lung injury (45). We did not see an up-regulation of IFNγ in the current study, this is likely due to the timing of lung collection, given that we measured inflammatory proteins in the lungs collected after 24 h of ventilation. IFNγ is seen to increase transiently in response to the initiation of ventilation with levels reducing over a period of hours-to-days after initial increase (46). TNFα is also found to be released in response to VILI in preterm neonates (45) however, pre-treatment with betamethasone, as was given in the current study, can prevent an increase in TNFα (47). UCB treatment in AG lambs attenuated immune cell infiltration into lungs but did not prevent the increase in IL-8 in either AG or FGR lambs. Moreover, UCB induced a 1.6-fold increase in lung IL-21 in AG and FGR lambs. IL-21 is a pro-inflammatory cytokine that promotes M2 "repair" to M1 "classically activated" macrophage phenotype, as well as increasing $CD4^+$ and $CD8^+$ T cell production (48), thus promoting inflammation. Persistence of inflammatory markers upregulated in response to mechanical ventilation in the current study are in contrast to previous reports where administration of placental stem cells increased expression of anti-inflammatory cytokines and reduced markers of lung inflammation following hyperoxic injury, thereby preventing downstream fibrosis and normalizing lung morphology (49). Differences between the findings here and those of previous studies for cell efficacy may reflect differences in the timing of tissue collection, mode of lung injury, the stage of lung development, or indeed the cells administered. It is perhaps too early to speculate whether the large increase in pulmonary IL-21 in response to UCB cells is a reparative or damaging effect, but it is increasingly well-understood that stem cells can modify a reparative response via immunomodulatory actions. This, however, is contingent on the inflammatory environment at the time of cell administration; stem cells introduced into a highly inflammatory host inhibit the protective capacity of stem cells (50), and can, in some instances, result in stem cells themselves becoming pro-inflammatory (51). This is a research area that requires further characterization, particularly for the vulnerable fragile preterm lungs.

Consistent with previous findings, we observed suppression of septal crests density following ventilation (16), and elastin distribution became diffuse along the alveolar wall; in AG lambs elastin density was significantly reduced and a similar (non-significant) trend was seen in lungs from FGR lambs. Ventilation in neonatal sheep and mice induces an upregulation of elastin production, but not the regulators of elastin assembly, leading to disordered accumulation of elastin along the alveolar walls (52). The qualitative changes we observed are likely a precursor to abnormal elastin deposition, highlighting the importance of treating in this acute period, before structural changes. Collagen, elastin and α-smooth muscle actin are essential structural components of the lung (53), and perturbations to the density and distribution of these factors will alter the mechanics of the lung. Twenty-four hours of ventilation in our preterm lambs decreased α-smooth muscle actin density in the FGR cohort compared to unventilated controls. Long-term (1 month) of ventilation increases α-smooth muscle actin (54), and thus we may have observed a transient decrease in this study, before a secondary compensatory increase. The mechanisms underlying the decreased α-smooth muscle actin in this current study are unknown, however, it is interesting to speculate on the possible role of nitric oxide (NO). In culture, increased NO reduces smooth muscle production, whilst inhibition of NO results in smooth muscle accumulation (55). It is well-accepted that growth restriction impairs NO handling (56, 57), and we have shown decreased content and altered distribution of the NO precursor, eNOS, following 2 h of ventilation (58). However, exposure to hyperoxia in the first day of life may increase local NO production due to impaired NO handling in FGR newborns, thereby resulting in inhibition of smooth muscle production.

Septal crest density is vital for increasing the surface area available for gas exchange. Ventilation induced a decrease in septal crest density in AG and FGR lambs, which is representative of simplification of the airways, and this is a hallmark of bronchopulmonary dysplasia (59). UCB was protective for septal crest density in the lungs of AG lambs, but not the FGR lambs. Despite this, we observed an improvement of injury heterogeneity in both AG and FGR lambs with treatment. Therefore, UCB may also promote, via paracrine mechanisms, surfactant production to reduce atelectasis.

Strikingly, there was an increase in the collagen to tissue ratio after UCB administration in FGR lambs. In previous studies, UCB-derived mesenchymal stem cells (MSCs) have increased fibroblast formation compared to those sourced from adipose tissue or bone marrow (13, 60). Despite this, no previous studies observed increased collagen; and UCB-MSCs or mononuclear cells administered to mice with VILI did not alter levels of TGF-β, a regulator of collagen production, or collagen content 14 days after cell administration (15, 50). However, to the authors' knowledge, there are no other studies specifically aimed at determining the efficacy of cell treatment in a growth restricted population. Whilst it is possible that the increase in collagen in FGR lambs in the current study is transient, given the relationship between collagen deposition and fibrotic disease, this relationship requires additional research.

Alveolar epithelial cells are a key source of increased cell proliferation following ventilation induced lung injury (61). Interestingly, cell proliferation was significantly increased in ventilated AG lambs but reduced in FGR ventilated lambs. FGR

is linked with lower levels of growth factors and decreased pulmonary cell growth in culture (11). We have previously shown that glucocorticoids reduce cell proliferation, both in AG and FGR fetuses (38) but since all groups received betamethasone, this is unlikely to have caused the difference observed here. It is more likely that the growth restricted lung does not respond to stretch by inducing proliferation, a well-established mechanism in the lungs of AG infants. Indeed, another key stretch response, the baroreflex response, is significantly attenuated in growth restricted fetuses (62), suggesting a possible decreased responsivity to this critical form of stimulus. Overall, these unexpected findings re-emphasize that even though ventilator requirements and fetal histology was not different between groups, FGR lungs respond differently to ventilation compared to AG lungs, and these changes may underpin the increased vulnerability to injury and long-term morbidity in FGR offspring. UCB treatment did not improve cell proliferation in FGR lungs. The underlying physiology is unknown, however investigating which cells are proliferative in AG would be of interest.

Treatment with UCB in FGR ventilated lambs promoted blood vessel growth as evidenced by the increased VEGF and vessel number, a finding not seen in AG lambs. VEGF is a potent inducer of angiogenesis and decreased VEGF expression is seen in newborns with BPD. *In vitro*, both MSCs (63) and endothelial progenitor cells (64) promote angiogenesis, via increases in VEGF. It is possible that, given time, increased vascularization of the lung would promote restoration of alveolarization, given the known positive relationship between these two factors (65). It is interesting, but not immediately apparent, why VEGF was increased in FGR, but not AG ventilated lambs. It is known that hypoxia increases VEGF production, and placental insufficiency directly exposes the developing fetus to chronic hypoxemia, which may in turn, result in impaired or altered hypoxia sensing and handling, and response.

How stem cells exert a reparative benefit is still not fully understood, however several mechanisms are possible. They may migrate to areas of injury and release trophic factors to reduce inflammation and promote endogenous repair mechanisms or they may alter systemic immune-modulatory responses (66). We observed only small numbers of UCB cells within the lungs, suggesting that their main effect was not via cell engraftment, but rather a paracrine effect as expected. *In vitro*, MSCs demonstrated preferential migration to hyperoxia-injured lung tissue rather than control medium or healthy lung tissue (49), suggesting stem cells are specifically recruited to sites of injury. Intra-tracheal UCB administration provides direct access to the lung and may improve lung outcomes (35), however, given that intubation is increasingly infrequent in pediatrics (67), systemic administration of cells is more clinically relevant.

It is now well-established that a poor uterine environment has the potential to program disease in later life (68). Emerging evidence also suggests that subtle, sub-clinical alterations are present in the lung (69) at the time of birth, which not only changes the function of the organ but can also alter the response of the organs to additional insults. It is likely that the lung is similarly affected, where an altered response to injury in FGR as compared to AG offspring has been demonstrated in animal models (70) and humans (3). It follows that treatments also may have different therapeutic ranges in infants following a sub-optimal pregnancy, and therefore further research is required to determine how to best target therapies to this population.

Limitations

We administered the UCB cells at a dose of 25 million cells/kg, based on evidence that this level is neuroprotective, and gave this dose 1 h after birth. It is possible that this cell dose and timing is not optimal for treatment of the lungs of the FGR infant and highlights the need to consider the FGR population independent of AG preterm infants. As with many other therapeutics, the timing of stem cell administration is vital, and it is possible that delaying cell administration until after the primary inflammatory phase is more protective in the lung, as has been observed *in vitro* (51). Further studies should examine this possibility and also determine the effects of UCB on VILI, in AG and FGR animals, beyond the 24-h. This will offer a better understanding of how UCB may benefit functional and morphological outcomes and chronic lung disease. Finally, the FGR infant has multi-organ dysfunctions and, while we only examined lung outcomes in the current study, optimizing postnatal therapeutics for multiple organs, including the lung, brain and cardiovascular system, must be considered. Treatment strategies to improve neurological structure and function are now being examined, including cell therapies. The current study suggests that we need additional targeted research to determine the interaction between organ systems, possible developmental programming and postnatal treatments, such as cell therapies.

Conclusion

FGR newborns have an increased risk of bronchopulmonary dysplasia and, whilst there is currently no cure for FGR or lung disease in this vulnerable cohort, UCB stem cells have shown potential therapeutic benefits in preterm infants. Here we sought to determine if UCB cells would also be beneficial for growth restricted preterm newborns. Our results have demonstrated that UCB shows promising anti-inflammatory benefits for treating ventilation induced lung injury in appropriately grown newborn lambs. However, UCB treatment was not equally effective for FGR infants, where it promoted angiogenesis, did not reverse detrimental changes in lung structure, and increased collagen and its precursor αSMA, which may be injurious. Interestingly, the pulmonary response to cell administration was differentially regulated in AG and FGR lambs, wherein UCB increased VEGF and decreased cell proliferation in FGR lambs only, however, whether this would be beneficial or not for the future of the offspring is yet to be determined. Our study is the first to highlight that the FGR infant responds differently to cell therapy, and these results suggest that developmental programming *in utero* needs to be considered when giving postnatal treatments.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in article/**Supplementary Material**.

ETHICS STATEMENT

Ethical approval for all experimental procedures utilized in this project was granted through the Monash Medical Centre Animal Ethics Committee (approval number MMCA2014-04).

AUTHOR CONTRIBUTIONS

BA, AM, GP, and SM conceived and designed the analysis. BA, HY, AM, CM, MC-M, YP, AS, GJ, GP, and SM collected and contributed to the data. BA and HY performed the analysis and BA, HY, AM, CM, MC-M, YP, AS, GJ, GP, and SM wrote the paper.

FUNDING

This work was supported by NHMRC Project Grant APP1083520 and the Victorian Government's Operational Infrastructure Support Program.

ACKNOWLEDGMENTS

We would like to acknowledge the assistance of Ilias Nitsos, Tamara Yawno, and Michael Fahey who assisted with the animal studies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00086/full#supplementary-material

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