VASCULAR DYSFUNCTION BEYOND PATHOLOGICAL PREGNANCIES. AN INTERNATIONAL EFFORT ADDRESSED TO FILL THE GAPS IN LATIN AMERICA, VOLUME II

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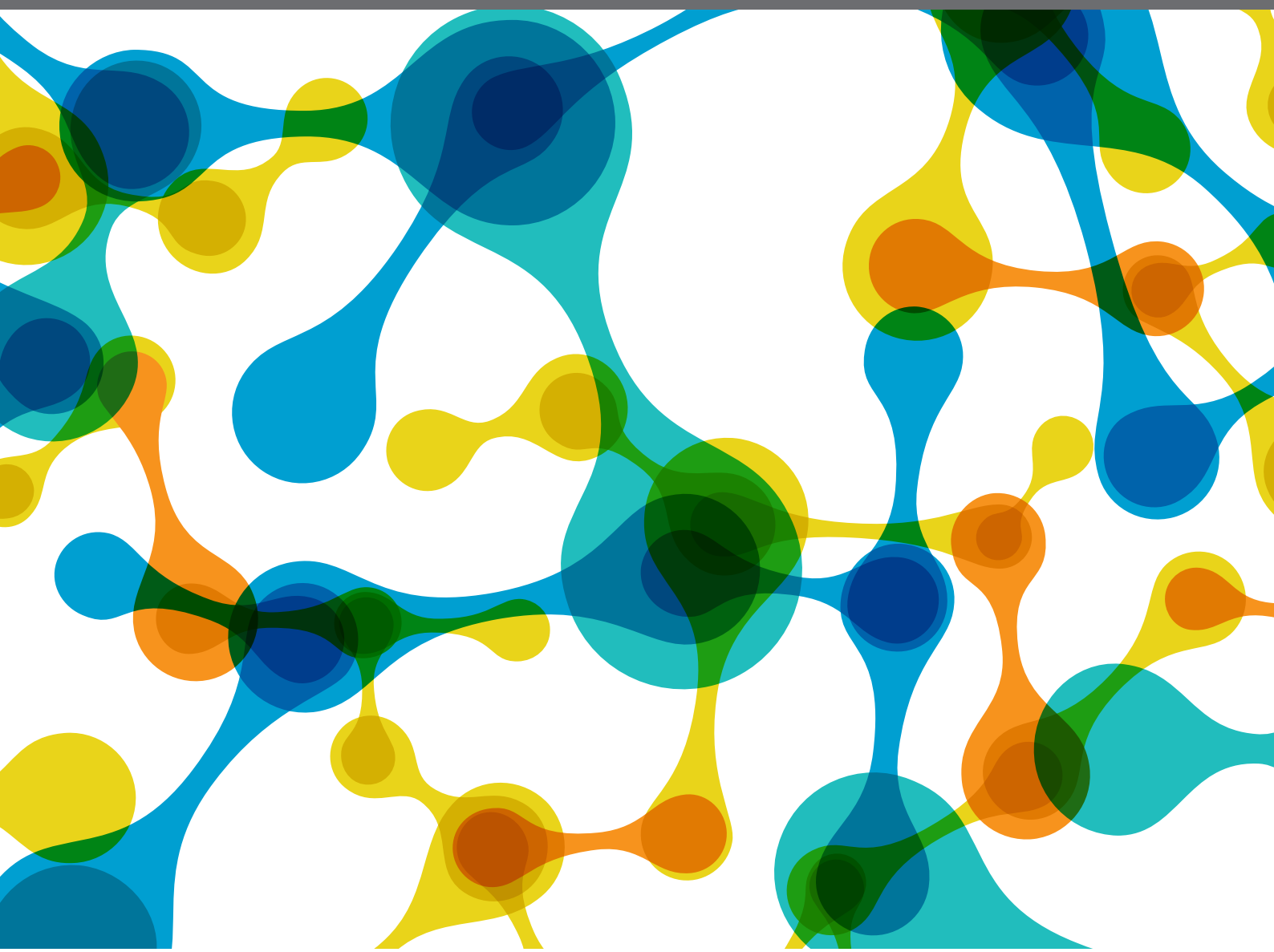

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VASCULAR DYSFUNCTION BEYOND PATHOLOGICAL PREGNANCIES. AN INTERNATIONAL EFFORT ADDRESSED TO FILL THE GAPS IN LATIN AMERICA, VOLUME II

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Editorial: Vascular dysfunction beyond pathological pregnancies. An international effort addressed to fill the gaps in Latin America, Volume II

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Editorial on the Research Topic
Vascular dysfunction beyond pathological pregnancies. An international effort addressed to fill the gaps in Latin America Volume II

Recent evidence suggests that vascular changes associated with pregnancy complications, such as preeclampsia; gestational diabetes; growth restriction; autoimmune diseases; among others, affect the function of the maternal and offspring vascular systems after delivery and may be extended until adult life (Giachini et al., 2017; Dayan et al., 2018; Honigberg et al., 2019). Furthermore, since the vascular system contributes to systemic homeostasis, defective development or function of blood vessels predisposes both mother and infant to future risk for chronic disease (Davis et al., 2012; Phipps et al., 2019).

In Latin American countries, the rate of morbi-mortality due to pregnancy complications and cardiovascular diseases has a higher incidence than in high-income countries (HIC) (Lopez-Jaramillo, 2009; Cuevas et al., 2014). But, the biomedical investigation into the cardiovascular consequences of pregnancy coming from Latin American countries still falls short of what would be expected considering the magnitude

of those diseases. Although there are obvious deficiencies in terms of economies and scientific infrastructure between HIC and Latin American countries, strength in terms of scientific productivity in this field could be underestimated. Which is associated with language limitations and publication in journals not indexed in major citation databases resulting in low-impact publications (Van Noorden, 2014). Unfortunately, an investigation performed in LIC or MIC receives minor citation even if it is published in high-impact journals (Meneghini et al., 2008). As a result, we could speculate that potentially unique features of vascular disease associated with pregnancy complications can be unnoted in the global scientific community.

Trying to overcome this potential bias in the literature, in collaboration with the Red Iberoamericana de alteraciones Vasculares Asociadas a TRastornos del EMbarazo (RIVA-TREM), we generate the first volume of our Research Topic in Frontiers in Physiology. We published 13 articles, with the valuable contribution of 60 authors. Since then, our scientific production has been viewed more than 60K times, and an increasing number of downloads from researchers worldwide. Then, we decided to continue encouraging Latin American Researchers in vascular biology to continue contributing to a better understanding of vascular dysfunction associated with pregnancy diseases and show the gaps in the literature, to overcome this hidden effect of our scientific production. Therefore, it is a pleasure to present volume II, with more authors (n = 121) and manuscripts (n = 19) than volume I. In the following sections, we highlight the relevance of those publications (Blanco et al., 2022).

One important event that massively affected the Latin American countries and the entire world was the pandemic of COVID-19. In this issue, Ayala-Ramirez et al. (10.3389/fphys.2022.785274) carry out a broad and concise review of the evidence about the compromise in pregnancy due to SARS-CoV-2 infection and COVID-19. In addition, they analyze alterations in cardiovascular adaptations and changes in the renin-angiotensin-aldosterone system during pregnancy. Authors emphasize information on pregnant women the information on pregnant women in Latin America and the need to generate more excellent knowledge about the impact of the pandemic on pregnancy in these countries.

The concern about the impact of non-transmissible maternal conditions on pregnancy and offspring, such as obesity and diabetes mellitus, has also been carefully considered by the contributors' authors. In this regard, gestational diabetes mellitus is a complex multifactorial disorder with a strong genetic predisposition. Higa et al. (10.3389/fphys.2021.760251) reviewed the effect of gestational diabetes mellitus as a trigger of fetal programming associated with a high risk of metabolic and cardiovascular diseases. Since the high diabetes prevalence before and during pregnancy and the increasing prevalence of obesity in pregnant women, the programming effect of those conditions extends the awareness for the next generations. This timely review provides information on studies of both human and experimental models addressing putative mechanisms involved in the fetal programming of the heart damage associated with maternal diabetes. In addition, Ortega-Contreras et al. (10.3389/fphys.2022.769924) describe different single nucleotide variations on genes related to different pathophysiological mechanisms associated with gestational diabetes mellitus. This analysis includes genes associated with alterations in transcription factors, hormones, membrane proteins, enzymes, growth factors, and others that may disturb insulin production and excretion or impair/modulate endothelial function.

Genetic factors and other parameters, such as hormones, are involved in maternal diabetes and placental dysfunction mechanisms. Louis et al. (10.3389/fphys.2021.76592) present original data on how the melatonin hormone prevents oxidative stress in maternal blood and placenta of women with diabetes. The authors found that melatonin levels were higher in maternal blood but reduced in the placental villus of women with type 2 diabetes. These findings were associated with a high rate of placental superoxide release, as well as in blood mononuclear cells of diabetic women, which was significantly reduced by melatonin. In addition, melatonin reduces placental and peripheral mononuclear cells' apoptosis. In addition, in the field of melatonin, Valenzuela-Melgarejo et al. (10.3389/fphys.2021.767684) evaluated the evidence for the potential beneficial effects of this molecule during hypertension. These authors describe that melatonin supplementation reduces blood pressure levels, prevents oxidative stress, improves antioxidant systems, and reduces Soluble Fms-Like Tyrosine Kinase 1 (sFlt-1) levels. Hence, melatonin can prevent endothelial damage in the placenta and may restore umbilical and uterine blood flow affected in pathological pregnancies, including preeclampsia or gestational diabetes.

Araujo-Silva et al. (10.3389/fphys.2021.701767) showed that maternal diabetes and obesity modulate immunological and metabolic changes associated with an increased risk of growth and development abnormalities in offspring in a murine model. These results are of great interest due to the possibility of being translated to human pregnancy.

Another essential contributor to maternal morbidity within the Latin American population is preeclampsia. The pathophysiology of preeclampsia and its complications are still elusive. In this field, Ayala-Ramirez et al. (10.3389/fphys. 2021.708824) reported an increase in pro-apoptotic molecules, such as Fas ligand (FasL) and TNF-Related Apoptosis-Inducing Ligand (TRAIL). These augmentations were observed in extracellular vesicles derived from cultures of placental explants from pregnancy affected by preeclampsia. Also, they showed how these extracellular vesicles had a high capacity to induce apoptosis in an *in vitro* model. This study explored how placental-derived extracellular vesicles might participate in the pathophysiology of preeclampsia. The results raise interesting questions about mechanisms involved, like trophoblast, immune

and vascular endothelial cells. Whereas, Torres-Vergara et al. (10.3389/fphys.2021.805082) hypothesized that another pro-apoptotic marker, sFlt-1, maybe a protective rather than deleterious on the cerebrovascular bed. They proposed that the elevation of sFlt-1 observed in preeclampsia could have a protective effect on the blood-brain barrier.

Another important aspect is to reveal biomarkers to be used to predict preeclampsia. Corominas et al. (10.3389/fphys.2021.785219) found that serum uric acid levels are increased in all forms of preeclampsia. However, the time of the rise of uric acid levels depended on the severity of the disease. In addition, their results revealed that uric acid levels do not increase in normotensive gestations with intrauterine growth restriction (IUGR), allowing a differential diagnosis between IUGR with and without preeclampsia. They found that uric acid levels less than 1.5 are a helpful parameter with a substantial exclusion value and high sensitivity for women who are not expected to develop preeclampsia. Implementing this low-cost test as a routine medical practice for all pregnant women would improve the use of resources in low-income countries. A more sophisticated analysis describes that microRNAs also have potential use as a biomarker for preeclampsia. Luizon et al. (10.3389/fphys.2021.678184) identified as upregulated the miR-204-5p in second-trimester plasma samples of women who eventually developed preeclampsia. Also, they showed through bioinformatics tools how miR-204-5p regulates the expression of genes potentially linked to preeclampsia. Therefore, they propose this microRNA as a biomarker of preeclampsia.

Several contributions are included in this issue regarding the pathophysiological mechanisms by which preeclampsia elicits trophoblastic cell and placental dysfunction. Medina et al. (10.3389/fphys.2021.774095) showed that aquaporin (AQP)-9 can function as a lactate transporter and have a role in energy metabolism or as a reactive oxygen species (ROS) scavenger. Authors propose that the lack of functionality of AQP9 may impair the placenta's lactic acid utilization, promoting ROS accumulation and adversely affecting the survival of the trophoblast cells. It also confirmed the presence of two mitochondrial subpopulations, which exhibit different morphologic and metabolic states, and revealed that AQP9 localized in the heavy/large mitochondria of the villous trophoblast cells.

Hence, other AQPs might be enrolled in a range of trophoblastic/placental activities during pregnancy. Dos Passos Junior et al. (10.3389/fphys.2021.696495) investigated the role of AQP-3 in trophoblastic cell migration. They show that tumor necrosis factor α (TNF-α) negatively modulates AQP-3 in placental explant and trophoblastic cells, reducing cell migration. Yet, they used an experimental model of spontaneously hypertensive rats (SHR) to show that placental levels of TNF-α were higher in the hypertensive group, whereas AQP-3 expression was reduced.

In addition, Troiano et al. (10.3389/fphys.2021.760237) used SHR rats to investigate the role of caveolae/caveolin-1 (Cav-1) on endothelial nitric oxide synthase (eNOS) activation during pregnancy. They found that in normotensive and hypertensive pregnancy, eNOS activation augment due reduced Cav-1/eNOS interaction favoring nitric oxide production in the aorta and mesenteric resistance arteries.

Mendes Silva et al. (10.3389/fphys.2021.766382) showed original contribution in a scientific area not yet analyzed in detail in Latin American countries, such as the infection of Group B *Streptococcus* (GBS) during pregnancy and its negative consequences on the function of trophoblast and endothelial cells. They use an *in vitro* approach in which chorionic villi explant, HTR-8/SVneo trophoblastic cell, and Ea. hy926 endothelial cell line were exposed to non-infected GBS. Also, they treated those cells with pentacyclic triterpene uvaol (urs-12-ene-3,28-diol), a component of the oliva oil with anti-inflammatory effects. Their findings demonstrated that GBS causes placental inflammation and oxidative stress, reducing trophoblast invasion of endothelial cells and increasing CXCL-8 and IL-6. These key factors participate in vascular dysregulation observed in several diseases. Since uvaol treatment prevented most of the GBS-provoked changes, they suggest that this molecule may be prophylactic for the potentially harmful effects of GBS infection for both the mother and the fetus.

Transmissible infections impose a maternal stress environment during pregnancy. Maternal stress refers to a set of chronic or acute injuries experienced by the mother during the pregnancy. One recognized model of maternal stress is the early alcohol consumption during pregnancy, a condition related to the development of fetal alcohol syndrome spectrum. The mechanism can be directly associated with fetal damage or indirectly through placental dysfunction. Gualdoni et al. (10.3389/fphys.2021.815760) provide bibliographic evidence of the harm imposed on placental structure and function secondary to maternal alcohol consumption. The authors propose a mechanism by which alcohol can disrupt the vascular development of mouse placenta by impairing vascular endothelial growth factor (VEGF) and its downstream effectors such as eNOS and metalloproteinases.

Costa et al. (10.3389/fphys.2022.787617) bring to light that the immune system might be bridging maternal stress and offspring vascular dysfunction. They acknowledge a disrupted modulation of the immune system by the maternal hypothalamic-pituitary-adrenal axis rise in response to maternal stress. In response to maternal stress, monocytes, natural killer cells, T cells, B cells, and antigen-presenting cells can be affected, contributing to the immune response-induced vascular dysfunction. This is characterized by an enhanced renin-aldosterone-angiotensin system, reactive oxygen species (ROS) accumulation, and toll-like receptor activation.

Maternal immune system dysfunction is also related to antiphospholipid syndrome (APS), an autoimmune disorder that

is characterized by pregnancy morbidity or thrombosis and persistent antiphospholipid antibodies (aPL). These antibodies can bind to the endothelium and induce endothelial activation, which is evidenced by the expression of adhesion molecules and the production of ROS and subsequent endothelial dysfunction marked by decreased synthesis and release of nitric oxide. Velasquez et al. (10.3389/fphys.2021.764702) revealed differences in endothelial activation and dysfunction among the different groups of patients with APS, which should be considered in future studies to evaluate new therapies, especially in refractory cases. On the other hand, Rodriguez et al. (10.3389/fphys.2021.706743) studied the impact of aPL from different patient populations on endothelial cell mitochondrial function, activation of the mTOR and autophagy pathways, and cellular growth. They found that aPL induces cellular stress evidenced by mitochondrial hyperpolarization and increased activation of the mTOR and autophagic pathways, which may play a role in the pathogenesis of obstetric APS.

Finally, Accialini et al. (10.3389/fphys.2021.667367) demonstrated that anandamide, a member of the endocannabinoid system, exerts a differential effect on prostaglandins concentration and NO bioactivity. This regulatory system is differentially involved in the onset of labor depending on the type of delivery, vaginal or cesarean section.

Therefore, we encourage the reader to analyze in detail the evidence in those manuscripts, and we invite you to discuss further, criticize, replicate results and learn from Latin American researchers. This invitation would help spread scientific research from this part of the world and overcome scientific bias in the international literature with extensive evidence from HIC.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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ORIGINAL RESEARCH
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Anandamide Exerts a Differential Effect on Human Placenta Before and After the Onset of Labor

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The onset of labor involves the action of multiple factors and recent reports have postulated the endocannabinoid system as a new regulator of this process. Our objective was to study the role of anandamide, one of the main endocannabinoids, on the regulation of placental molecules that contribute to the onset of labor at term. Placental samples were obtained from patients with laboring vaginal deliveries and from non-laboring elective cesarean sections. Vaginal delivery placentas produced higher prostaglandins levels than cesarean section samples. Besides, no differences were observed in NOS basal activity between groups. Incubation of vaginal delivery placentas with anandamide increased prostaglandins concentration and decreased NOS activity. Antagonism of type-1cannabinoid receptor (CB1) did not alter the effect observed on NOS activity. Conversely, incubation of cesarean section placentas with anandamide reduced prostaglandins levels and enhanced NOS activity, the latter involving the participation of CB1. Furthermore, we observed a differential expression of the main components of the endocannabinoid system between placental samples, being the change in CB1 localization the most relevant finding. Our results suggest that anandamide acts as a modulator of the signals that regulate labor, exerting differential actions depending on CB1 localization in laboring or non-laboring term placentas.

Keywords: anandamide, prostaglandins, nitric oxide synthase, placenta, labor

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INTRODUCTION

The onset of term human labor involves the action of a variety of factors that enables the transition from uterine quiescence to contractility. Indeed, an accurate interplay among different mediators produced by the placenta, mother, and fetus is necessary to trigger the initiation and progression of labor. In the last years extensive research has attempted to elucidate the factors involved in the mechanism of parturition. In line with this, several reports have postulated the endocannabinoid system (ES) as a new member of the complex range of factors that regulate the onset of labor. The ES is a signaling system that comprises the endogenous bioactive lipids called endocannabinoids (ECs); the cannabinoid receptors (CBs) and the enzymes involved in their metabolism. It was demonstrated that, in human gestation at term, plasma concentration of anandamide (N-arachidonoylethanolamine, AEA), one of the most extensively studied endocannabinoids, is higher in laboring than in non-laboring women (Habayeb et al., 2004). Furthermore, the human placenta

produces higher levels of AEA compared to other reproductive tissues (Marczylo et al., 2010). After its synthesis, AEA can act as an endogenous ligand of the CBs, which are inhibitory G protein coupled receptors (GPCRs) (Howlett, 2005). Particularly, type-1 CB (CB1) has been localized in human term placentas (Park et al., 2003). Appropriate desensitization of CB1 is a critical process in its regulation and it may occur by endocytosis (Rozenfeld, 2011; Wickert et al., 2018). Lipid raft and caveolae, which are plasma membrane subdomains, have been described as regulators of GPCRs signaling (Moffett et al., 2000). Particularly caveolin-1, which is the most relevant structural protein of caveolae (Patel and Insel, 2009), can act as a regulator of CB1 by internalizing this receptor via caveolae-related endocytosis (Lefkowitz, 1998).

During the third trimester, the contractile activity of the uterus gradually increases. Thus an increment in the factors associated with myometrial contraction is expected. It is well-known that prostaglandins (PGs) play a pivotal role in the process of labor since they stimulate myometrium contractility; cervical ripening and membrane rupture (Challis et al., 1997, 2002, 2009). Several factors are known modulators of placental PGs production (Carbillon et al., 2000; Keelan et al., 2000; Challis et al., 2001), and in the last years mounting evidence suggest that the ECs are also involved in its regulation (Mitchell et al., 2016).

Initiation of labor also involves profound hemodynamic changes. The placenta capability to produce vasoactive mediators and therefore to regulate placental circulation is cornerstone in this process (Myatt, 1992). The uterine contractions that occur during labor are associated with intermittent utero-placental perfusion, suggesting that changes in the level of factors controlling blood flow would contribute to the triggering of labor (Cindrova-Davies et al., 2007). Among these factors, nitric oxide (NO) is a well know auto/paracrine vasodilator agent that contributes to the reduction of the vascular resistance and the modulation of the uteroplacental and fetal circulation (Sladek et al., 1997). Nitric oxide production and NOS activity are strongly regulated by multiple factors. Particularly, AEA can stimulate or inhibit NOS activity and/or NO production through CBs activation depending on tissue context and cell type (Cella et al., 2008; Sordelli et al., 2011, 2012; Aban et al., 2013).

Even though the relationship between AEA action and the synthesis of PGs and NO has been previously demonstrated, its precise role as a regulatory molecule of the placental signals related with the onset of human labor has not been clarified.

For the aforementioned, in the present work we aimed to elucidate the participation of the ES in the regulation of PGs production and placental NOS activity in human placenta at term.

MATERIALS AND METHODS

Placental Samples and Tissue Collection

This study was approved by the Ethics Committee of the Center for Medical Education and Clinical Research "Norberto Quirno" (N° 684) and all patients signed informed consent.

Placental tissue was obtained from healthy normotensive women with uncomplicated pregnancies at term (37–40 completed weeks of gestation). Samples were divided into two groups: n = 28 were vaginal deliveries (VD) and progressed satisfactorily to a non-instrumented birth; n = 27 were non-laboring elective cesarean sections (CS). The characteristics of the patients are summarized in **Table 1**. Onset of labor was defined as the initiation of regular uterine activity, leading to cervical dilation and effacement. Women treated with oxytocin for induction of labor and women with intrapartum cesarean section were excluded from the study. All placental samples were collected and processed within 1 h after delivery. Following removal of blood vessels or clots, tissue was randomly cut into small pieces, repeatedly washed with saline solution to remove the excess blood, and finally either processed or stored at −80°C.

TABLE 1 | Clinical characteristics of the patients.

Parameter	Cesarean section (n = 27)	Vaginal delivery (n = 28)
Mean gestational age (weeks)	38.6 ± 0.2	40.0 ± 0.8
Mean maternal age (years)	34.8 ± 1.75	35.7 ± 1.9

Values are expressed as the mean ± SEM.
Patients from both groups have no previous history of hypertension, gestational diabetes, autoimmune diseases, multifetal gestation, or fetal anomalies.

Prostaglandins Concentration Measurement

Prostaglandin E2 (PGE2) and prostaglandin F2a (PGF2a) were measured in placental explants from VD or CS as described previously (Ribeiro et al., 2004; Cella et al., 2010). Briefly, tissues were incubated for 60 min in Krebs-Ringer bicarbonate buffer (5% v/v Krebs solution; 2.0 g/L glucose; 2.1 g/L $NaHCO_3$) at 37°C in a 5% CO_2 atmosphere. After incubation, placental tissues were used for total protein content measurement by Bradford technique. Supernatants were acidified and then PGs were extracted twice with ethyl acetate. Prostaglandins concentration was determined by radioimmunoassay. Sensitivity was 5–10 pg/ml, and values are expressed as pg PGs/mg of protein.

Incubation With AEA and Met-AEA

Fresh explants from VD or CS placentas were cultured with 10^{-5} mol/L, 10^{-7} mol/L, or 10^{-9} mol/L AEA (Biomol, Miami, United States) y el), or R-(+)-Methanandamide (Met-AEA, Sigma, St. Louis, United States), a stable AEA analogous, for 24 h in RPMI 1640 culture medium (Microvet, Buenos Aires, Argentina) supplemented with 10% v/v bovine fetal serum (Natocor, Córdoba, Argentina) and gentamicin (50 μg/ml). Additionally, as a positive control of PG synthesis, placental explants were incubated with LPS (lipopolysaccharide from *E. coli*, 1 mg/ml). After 24 h of culture, PGs concentration was determined as abovementioned.

NOS Activity Measurement

NOS activity was determined by the modified technique of Bredt and Snyder (Aban et al., 2013), which measures the conversion of [^{14}C]-arginine into [^{14}C]-citrulline. Tissues were homogenized in Hepes buffer (0.45 mmol/L $CaCl_2$; 25 mmol/L

valine and 100 mmol/L DTT) and then 0.12 mmol/L NADPH and 10 μmol/L [^{14}C]-arginine (0.3 μCi) (Cayman Chemical, Ann Arbor, United States) were added to the homogenate. Samples were incubated at 37°C in a 5% CO_2 incubator for 15 min and then centrifuged at 7,800 g for 15 min at 4°C. The supernatant was eluted in a DOWEX 50WX8 column ion-exchange resin (Na^+ form) (Biorad, Buenos Aires, Argentina). The column was washed three times with PBS 1X (137.0 mmol/L NaCl; 2.7 mmol/L KCl; 8.1 mmol/L Na2HPO4; 1.47 mmol/L KH2PO4; pH = 7.4) and the radioactivity of the eluates was quantified by liquid scintillation counting (Beckman). NOS activity was expressed as fmol of [^{14}C]-citrulline/mg of protein during 15 min.

Incubation With AEA

Fresh explants from VD or CS placentas were pre-incubated with 10^{-7} mol/L, 10^{-8} mol/L, or 10^{-9} mol/L AEA in Krebs buffer in a Dubnoff shaking incubator with a 5% CO_2 atmosphere at 37°C for 30 min. After culture, explants were homogenized in Hepes buffer as abovementioned.

Incubation With AM 251

Fresh explants from VD or CS placentas were pre-incubated with 10^{-7} mol/L AM251 (CB1 antagonist, Tocris Cookson Inc., Ellisville, United States) in Krebs buffer in a Dubnoff shaking incubator with a 5% CO_2 atmosphere at 37°C for 15 min. Then, 10^{-7} mol/L AEA was added and tissue was incubated 30 more minutes. After culture, explants were homogenized in Hepes buffer as mentioned before.

Western Blot

Samples were prepared as previously described (Aban et al., 2013, 2016) and protein concentration was measured by Bradford assay. Lysates of placental explants (100 μg of protein) were boiled for 5 min and then electrophoresed through a SDS–polyacrylamide gel. The resolved proteins were transferred onto a nitrocellulose membrane. After incubating the blot in blocking solution [5% w/v non-fat dry milk in 1% v/v Tween-PBS (T-PBS)], membranes were rinsed with T-PBS and incubated overnight at 4°C with the appropriate primary antibody: FAAH 1:150 (gift from Dr. Benjamin Cravatt), N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) 1:1,000 (Cayman Chemical Co., Ellsworth Road, MI, United States), CB1 1:250 (Biomol, Miami, United States), FLOTILIN 1:1,000 (Santa Cruz Biotechnology, California, United States), CAVEOLIN-1 1:500 (Santa Cruz Biotechnology), β-ACTIN 1:1,000 (Sigma, St. Louis, United States). Then the blots were rinsed with T-PBS and incubated with peroxidase-conjugate anti-Rabbit 1:10,000 (Jackson ImmunoResearch Laboratories, Sero-Immuno Diagnostics, INC, Tucker, GA, United States) at room temperature (RT) for 1 h. β-ACTIN was used as loading control. Proteins were visualized after incubation with enhanced chemiluminescence reagent (ECL, Sigma, St. Louis, United States) and light emission was detected by exposing the blots in an Image Quant 350 GE Healthcare. Protein content was quantified by densitometric analysis using ImageJ (NIH) program.

Fatty Acid Amide Hydrolase (FAAH) Activity

FAAH activity was determined as established by Paria et al. (1996) with minor modifications. Placental tissue was homogenized and protein quantification was performed by Bradford assay. Then, 100 μg of protein were incubated for 15 min at 37°C in buffer 50 mmol/L Tris pH = 8.5 containing AEA and radio-labeled [^{3}H]-AEA (0.05/μCi) (Perkin Elmer, Buenos Aires, Argentina). The reaction was stopped with a mixture of chloroform:methanol (1:1 v/v). After centrifugation, samples were resuspended in chloroform and spotted onto a thin chromatography Silica Gel 60 layer. The plate was exposed for 1 h in a saturated box with a mixture of acetic acid:ethyl acetate:hexane:distillated water (100:50:20:100 v/v). Standards of arachidonic acid (AA) and AEA were included as controls. Iodine was used to identify [^{3}H]-AA, which is one of the products of FAAH activity. Radioactivity on the plate was counted in a scintillation counter by scrapping off the corresponding spots. Enzyme activity was reported as nmol [^{3}H]-AA/mg protein/min.

Immunohistochemistry

Placental tissue was fixed in 4% v/v paraformaldehyde overnight at 4°C. Then, samples were dehydrated by graded ethanol washes and embedded in paraffin. Slides were deparaffinized in xylene and rehydrated by graded ethanol. Tissue sections were permeabilized with 0.1% v/v Triton X-100 and endogenous peroxidase activity was blocked with 3% v/v H_2O_2. Sections were first incubated in blocking solution (2% non-fat dry milk in PBS) for 1 h at RT and then with CB1 (Biomol, Miami, United States) primary antibody diluted 1:50 in PBS, in a moist chamber overnight at 4°C. Then, slides were rinsed thrice in PBS and incubated with specific biotinylated anti-rabbit secondary antibody (1:200) for 1 h at RT. After washing the slides, sections were incubated with streptavidin-peroxidase complex and then stained with 0.05% v/v 3,3'-diaminobenzidine and counterstained with hematoxylin. Negative controls were obtained in the absence of primary antibody. Digital images were acquired using a camera (Nikon Corporation) mounted on a conventional light microscope (Nikon).

RNA Isolation, Reverse Transcription, and Real-Time PCR

Total RNA was extracted using RNAzol reagent (Molecular Research Center, Cincinnati, United States) according to manufacturer's instruction and RNA concentration was quantified using a NanoDrop (Thermo Fisher Scientific). One microgram of total RNA was converted into cDNA using M-MLV reverse transcriptase (Promega, Buenos Aires, Argentina) and random primers. The mRNA levels of *Faah, Nape-PLD,* and *Cb1* were quantitatively measured by qPCR on RG6000 (Corvette) using EasyTaq DNA polymerase (TransGen Biotech, Buenos Aires, Argentina) and EvaGreen dye (Biotium). The program used was: 5 min at 95°C (one cycle), followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 1 min at 72°C for all primers. *Tata binding protein* (*Tbp*) was used as endogenous control, and negative control (no template) was included in all cases.

The mRNA level of each gene was corrected to the level of *Tbp* and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to the ratio produced in the CS samples. Primers (Invitrogen, Buenos Aires, Argentina) sequences were: *Faah* (Fw: GCACACGCTGGTTCCCTTC, Rv: GGGTCCACGAAATCACCTTTGA); *Nape-PLD* (Fw: TCCC TCCAATAGATGCGGTCCT, Rv: TCCTCCCACCAGTCCAAC TCAA); *Cb1* (Fw: CCGATACACTTGGCATTGAC, Rv: GA CCGGGGGTGTAAGAAGAAA); *Tbp* (Fw: CCCGAAACGCC GAATATAATCC, Rv: AATCAGTGCCGTGGTTCGTG).

Apical (MVM) and Basal (BM) Membrane Vesicles Isolation

Human placenta villi from VD and CS term placentas were processed for MVM and BM enrichment as previously described (Levi et al., 2016). After obtaining the MVM and BM vesicles, detergent-resistant membranes (DRMs) were isolated by sucrose gradient centrifugation as performed previously (Levi et al., 2016). Sucrose gradient fractions of MVM and BM were dialyzed against TNE buffer (10 mmol/L Tris, 200 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) overnight in order to eliminate sucrose and finally subjected to SDS–PAGE polyacrylamide gels for protein detection by Western blot analysis.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism Software (San Diego, CA). Data are expressed as the mean ± SEM and were analyzed using Student's *t*-tests or One-way ANOVA following Tukey's multiple comparison tests as post-test. Values of $p < 0.05$ were considered significant.

RESULTS

Analysis of PGs Concentration

To elucidate if differences in the basal production of PGs exist between non-labor cesarean section (CS) and vaginal delivery (VD) placentas, PGE and PGF2a concentrations were measured. Vaginal delivery placentas produce higher PGE and PGF2a levels than CS samples (**Figure 1A**), suggesting that labor modulating molecules regulate placental PGs production.

Since AEA plasma levels increase in women with spontaneous (Habayeb et al., 2004) and induced (Nallendran et al., 2010) labor, and we postulate this molecule as a relevant participant in this process (Accialini et al., 2020), the effect of AEA on placental PGs synthesis was analyzed. For this purpose, CS and VD placentas were incubated with different concentrations of AEA and then PGE and PGF2a concentration was measured. It is important to point out that the molecular products resulting from AEA degradation can be enzymatically converted into PGs and prostamides (Peiris et al., 2017). These products are indistinguishable by radioimmunoassay, leading to difficulties in differentiating them. For this reason, to determine if our observations were a consequence of AEA incubation instead of AEA degradation, CS and VD placentas were also incubated with Met-AEA, a non-hydrolyzable AEA analogous. Incubation with 10^{-5} mol/L AEA decreased PGE concentration in non-labor CS placentas (**Figure 1B**), while PGF2a maintained its levels unchanged (**Figure 1C**). Conversely, incubation of labor VD placentas with 10^{-5} mol/L and 10^{-7} mol/L AEA increased PGE (**Figure 1D**) and PGF2a (**Figure 1E**) concentration, respectively. The effect of Met-AEA incubation on PGs production remained identical to that observed with AEA for both placental groups. As a positive control of PGs synthesis, placentas were incubated with LPS, which resulted in an increase in PGE and PGF2a concentration in both CS and VD placentas (data not shown).

Characterization of NOS Activity

In line with our hypothesis that AEA acts as a key modulator in labor, previous results from our laboratory showed that AEA stimulates NOS activity through CB1 receptor in normal non-labor CS placentas (Aban et al., 2013). In the present work, NOS activity was analyzed comparing CS and VD placentas at term. Our results showed that there are no differences in the basal activity of this enzyme between groups (**Figure 2A**). However, incubation with 10^{-7} mol/L AEA produced a dual effect: while AEA enhanced NOS activity in CS samples, decreased this enzyme activity in VD placentas (**Figure 2B**).

In order to determine whether the dual effect that AEA has on NOS activity involves the participation of CB1, CS, and VD placental explants were co-incubated with AEA and AM251, a selective antagonist of CB1 receptor. Co-incubation with AEA + AM251 prevented the increment of NOS activity in CS samples (**Figure 2C**). Conversely, it does not alter the decrease observed in VD placentas (**Figure 2D**), suggesting that the inhibitory effect of AEA on NOS activity does not involve the participation of CB1.

Expression of Relevant Members of the Endocannabinoid System in Human Placenta at Term

The placenta expresses several components of the endocannabinoid system (ES) such as NAPE-PLD and FAAH (Aban et al., 2013), the main enzymes of AEA synthesis and degradation, respectively. Since AEA levels are modulated principally by these metabolic enzymes characterization of their expression was performed comparing CS and VD placentas at term. NAPE-PLD mRNA expression was lower in VD placentas compared to CS samples, while no differences were observed in its protein content (**Figure 3A**). Regarding FAAH, there were no differences in mRNA levels between groups, although a decrease in FAAH protein levels and enzymatic activity were observed in VD placentas (**Figure 3B**). This finding suggests that placental AEA concentration is higher in VD placentas. Lastly, CB1 receptor mRNA and protein levels were similar between groups (**Figure 3C**).

Analysis of CB1 Localization

Immunohistochemistry analysis revealed specific CB1 staining mainly in the apical membrane of the syncytiotrophoblast (STB) of CS placentas (**Figure 4A**). In VD samples, CB1 is localized in the STB cytoplasm (**Figure 4B**) although a slight staining is also observed in the basal membrane, suggesting that labor

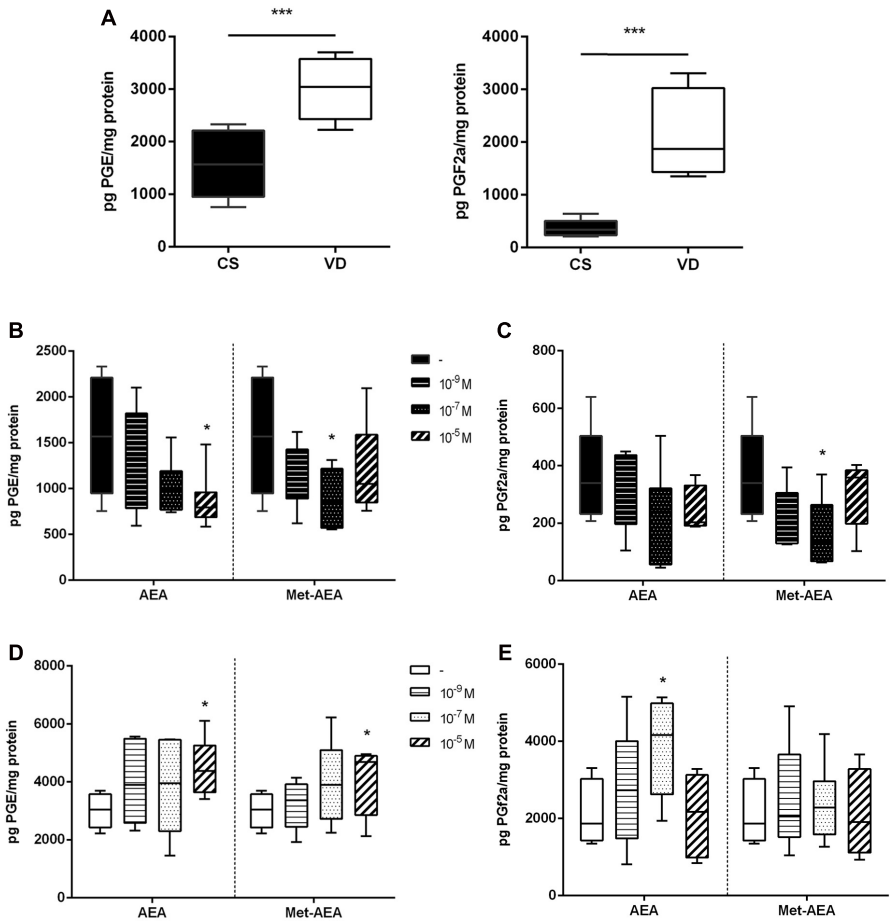

FIGURE 1 | Analysis of prostaglandins concentration in term placenta. **(A)** Basal prostaglandin E (left panel) and F2a (right panel) production in cesarean section (CS) and vaginal delivery (VD) placentas (***$P < 0.001$, $n = 10$). **(B)** Prostaglandin E and **(C)** prostaglandin F2a production measured after culturing CS placentas with different concentrations of AEA or Met-AEA (*$P < 0.05$ vs. control, $n = 10$). **(D)** Prostaglandin E and **(E)** prostaglandin F2a production measured after culturing VD placentas with different concentrations of AEA or Met-AEA (*$P < 0.05$ vs. control, $n = 10$).

modulates CB1 localization. Accumulating evidence shows that CB1 can localize within LRs, which are known regulators of GPCRs signaling (Moffett et al., 2000). Particularly, LRs have a functional effect on CB1 as they regulate its positioning on the membrane and its intracellular trafficking (Sarnataro et al., 2005) via caveolae-related endocytosis (Lefkowitz, 1998). In order to study whether CB1 localizes within LRs in the STB, detergent-resistant membranes (DRMs) technique was performed in CS and VD placentas. Although DRMs are different from LRs (Herlax et al., 2009), they constitute a useful approach to investigate the interaction between membrane subdomains and proteins, as we have previously described (Levi et al., 2016).

The STB is a polarized cell formed by an apical membrane (MVM) that contacts the maternal circulation and a basal membrane (BM) that interacts with the fetal capillaries. For this reason, enrichment in MVM and BM vesicles from CS and VD placentas was performed and then DRMs were isolated from these samples. To improve protein detection, elutes were grouped in five fractions (see methodology), with fraction number two carrying the LRs. Protein expression of CB1, CAVEOLIN-1 and FLOTILIN-1, which are specific markers of caveolae and LRs, respectively, were analyzed in DRMs fractions of MVM and BM obtained from CS and VD placentas. Western blot analysis revealed that CB1 is expressed mainly in the MVM of CS placentas in fractions different from fraction number 2, so that CB1 is not co-localized with CAVEOLIN-1 and FLOTILIN-1 (**Figure 4C**). A slight CB1 band is also evidenced in BM samples (**Figure 4C**). However, in VD placentas CB1 is expressed in the BM and co-localizes with CAVEOLIN-1 and FLOTILIN-1 (**Figure 4D**).

These results, together with the observation by immunohistochemistry, suggest that labor promotes a differential location of CB1, within LRs.

DISCUSSION

The placenta is a dynamic organ that changes as pregnancy progresses and during the transition from uterine quiescence to contractility at the time of labor (Vannuccini et al., 2016). In the

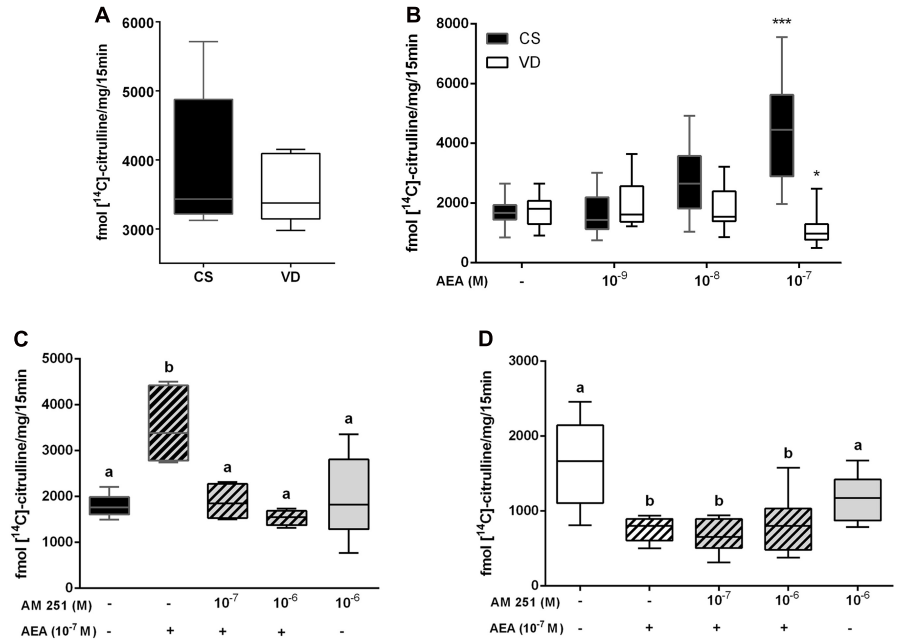

FIGURE 2 | Characterization of nitric oxide synthase (NOS) activity in term placenta. **(A)** Basal NOS activity measured in CS and VD placentas ($n = 8$). **(B)** NOS activity measured after culturing CS and VD placentas with different concentrations of AEA (***$P < 0.001$, *$P < 0.05$ vs. control, $n = 8$). **(C)** NOS activity measured after culturing CS placentas and **(D)** VD placentas with 10^{-7}M AEA and different concentrations of AM 251. Different letters represent significant changes ($P < 0.05$ vs. control, $n = 8$) and the plain gray bar shows that AM 251 has no effect *per se*.

last years several reports have suggested a strong relationship between the endocannabinoid system (ES) and pregnancy performance, as its deregulation may lead to pathological conditions such as preeclampsia (Aban et al., 2013) and preterm labor (Wang et al., 2008; Bariani et al., 2015). In this work we demonstrate that changes in the expression and localization of members of the ES occur with the onset of labor, and that anandamide (AEA) differentially modulates prostaglandins (PGs) synthesis and nitric oxide synthase (NOS) activity in the human placenta at term.

During the third trimester, there is a progressive increase in the contractile activity of the uterus and therefore an increase in factors associated with muscle contraction such as PGs, is expected. Our results showed that vaginal delivery (VD) placentas have higher PGE and PGF2a concentration than non-labor cesarean section (CS) placentas.

Anandamide has been postulated as a relevant participant in labor (Habayeb et al., 2004; Nallendran et al., 2010). Previous results from our laboratory showed that this endocannabinoid contributes to the modulation of the oxytocin system in human placenta at term (Accialini et al., 2020). In line with this hypothesis, we observed a dual effect of AEA on PGs production, which suggests that the action of AEA depends on the placenta's molecular context (labor vs. non-labor). Accordingly, Bariani et al. reported that AEA increases uterine PGF2a levels in a mouse model of preterm labor induced by LPS (Bariani et al., 2015). Furthermore, it was also reported that AEA upregulates Cyclooxygenase 2 (COX-2) expression and PGs synthesis in human fetal membranes as well (Mitchell et al., 2008), suggesting an important role of this endocannabinoid in the trigger of parturition.

Our observation on PGs synthesis led us to speculate if molecules involved in myometrial quiescence are also regulated by AEA. Nitric oxide (NO) is responsible for maintaining uterine quiescence (Ledingham et al., 2000) and for regulating placental corticotropin releasing hormone (CRH) release during pregnancy (Roe et al., 1996). This modulating action is important since CRH has been postulated as a molecular clock that determines the length of pregnancy and the time of birth (McLean et al., 1995). Moreover, NO is also one of the main molecules that regulate placental blood flow. In the present work we analyzed NOS activity, and no differences were found between VD and CS samples. In line with these results, it was reported that eNOS expression is similar in both CS and VD placentas (Kakui et al., 2003), and that NOS activity is higher in term CS placentas when compared to the myometrium of the same patient (Al-Hijji et al., 2003). However, it is important to point out that *in vitro* NOS enzymatic activity may not faithfully reflect the *in vivo* NO production because several co-factors participate in its synthesis. Accordingly, NO levels are modulated by several factors, including ECs. We previously demonstrated that AEA contributes to the fine regulation of NO levels in rat placenta (Cella et al., 2008). In the present study we observed that AEA exerts a differential effect on NOS activity. We speculate that during pregnancy, AEA increases NO levels to maintain uterine quiescence and to contribute to a vasodilatation state necessary for adequate oxygen and nutrients supply to the fetus. Conversely, a decrease in NOS

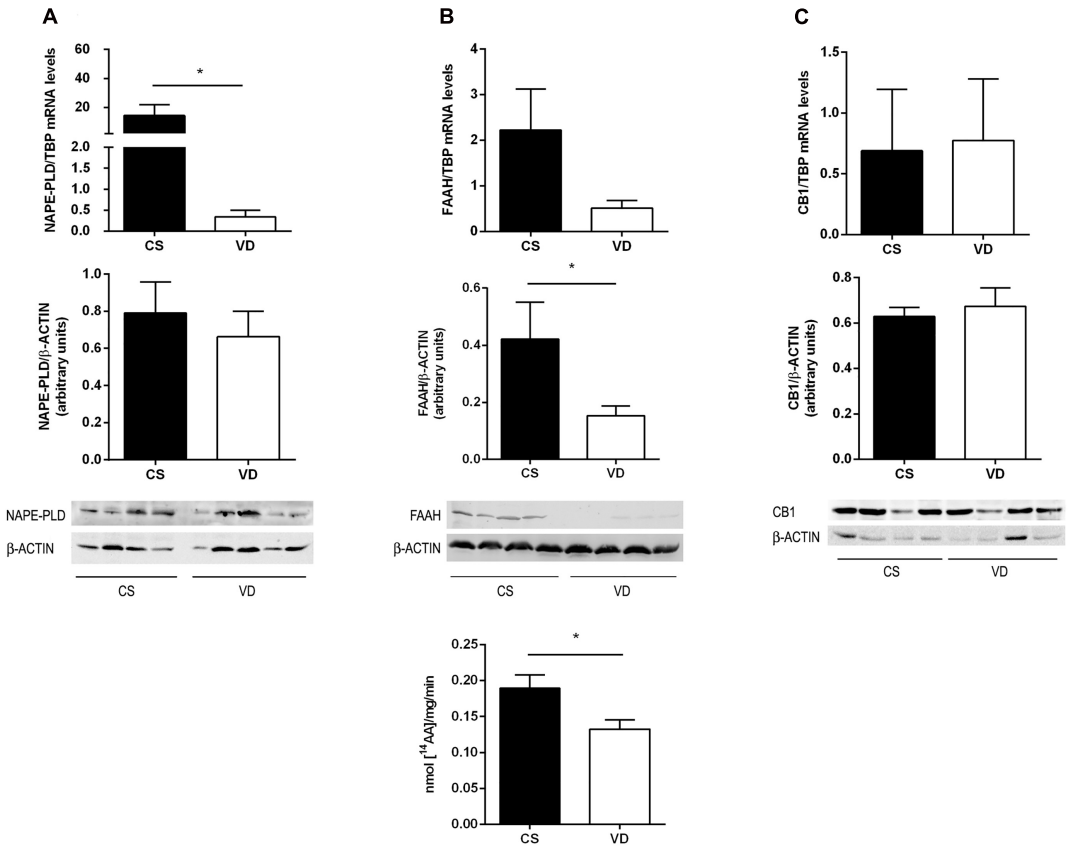

FIGURE 3 | Expression of endocannabinoid system in term placenta. **(A)** NAPE-PLD mRNA levels and protein content, **(B)** FAAH mRNA levels, protein content and enzymatic activity, and **(C)** CB1 mRNA levels and protein content, measured in CS and VD placentas. Representative immunoblots of protein content are shown in the lower panel (*$P < 0.05$, $n = 4$–5).

activity in VD placentas contributes to releasing the myometrium from relaxants molecules in preparation to receive contractile signals that stimulate the onset of parturition. Besides it would also be critical during labor for preventing excessive bleeding during parturition.

Our results suggest that AEA plays an important role in labor, regulating factors that in turn stimulate either myometrial quiescence or contractility. Furthermore, its action seems to depend on the molecular context of the placenta.

Given that increased AEA levels are considered one of the signals that contribute to the onset of labor, and since the expression of the ES in the placenta during this process remains unknown, we evaluated the expression of members of the ES which are known to be important regulators in this tissue. In agreement with a previous report (Park et al., 2003), we observed that VD placentas express lower FAAH protein levels than CS placentas. In addition, we evidenced a decrease in FAAH enzymatic activity in VD samples, suggesting that placental AEA levels increase with parturition, possibly contributing to the overall increment in AEA tone. The decrease observed in NAPE-PLD mRNA expression in VD samples may be a compensatory mechanism for the presumed rise in placental AEA tone.

Several reports have demonstrated the relevance of CB1 signaling in the regulation of parturition. In fact, defective CB1 signaling in mouse has been associated with preterm onset of labor (Wang et al., 2008). Accordingly, Acone et al. (2009) detected a decrease in CB1 protein levels between non-laboring and laboring placentas. In the present study we did not detect differences in CB1 mRNA and protein levels between samples. The differences with the above-mentioned reports can be attributed to the selection criteria and/or the type of samples included in the study. The distribution of GPCRs on the cell surface and their internalization are regulated by different mechanisms, being the trafficking through Lipid rafts (LRs) a well-described one. A subset of LRs found in cell surface invaginations is called caveolae, in which caveolins (CAVs) are the most relevant structural proteins (Patel and Insel, 2009). Particularly, LRs have a functional effect on CB1 as they regulate not only its partitioning on the membrane and thus the accessibility to the ligand, but also its intracellular trafficking (Sarnataro et al., 2005) via caveolae-mediated endocytosis (Lefkowitz, 1998; Wickert et al., 2018). Due to the importance of CB1 in the regulation of parturition, and since differences in its expression were not observed between placental samples

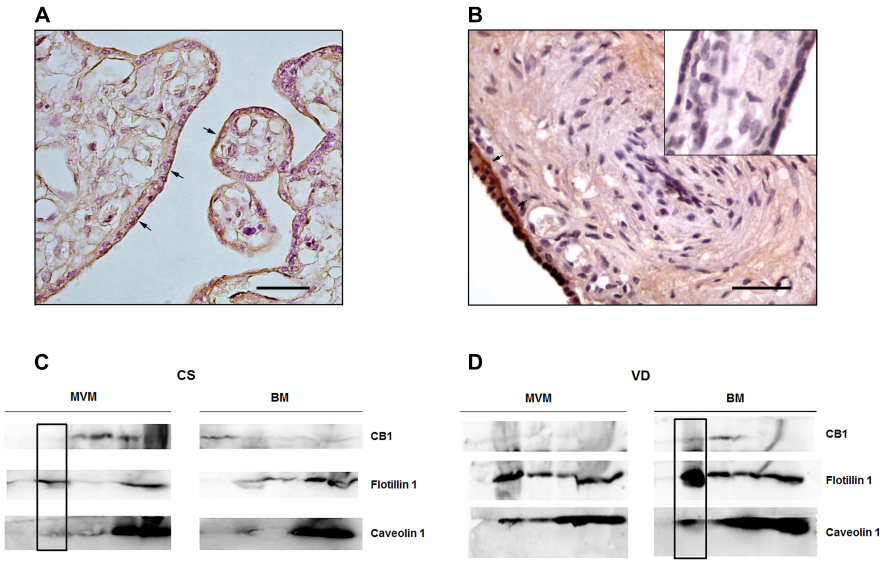

FIGURE 4 | Analysis of CB1 localization in the syncytiotrophoblast of term placenta. Immunolocalization of CB1 in representative sections of placental villous from **(A)** CS placentas and **(B)** VD placentas. The arrows indicate specific CB1 staining and the inset shows the negative control. Magnification: 40×. Scale bar: 20 μm. **(C)** Differential distribution of CB1, Flotillin 1 and Caveolin 1 in sucrose gradient fraction of apical membrane (MVM) and basal membrane (BM) from CS placentas. CB1 is expressed in MVM in fractions different from fraction number 2. The inset shows that CB1 does not co-localize with Flotillin 1 and Caveolin 1 (n = 3). **(D)** Differential distribution of CB1, Flotillin 1 and Caveolin 1 in sucrose gradient fraction of MVM and BM from VD placentas. The inset shows that CB1 co-localizes with Flotillin 1 and Caveolin 1 in BM (n = 3).

that may explain AEA's dual effects, we evaluated CB1 immunolocalization. We observed specific CB1 staining mainly in the apical membrane of the STB of CS placentas, while in VD samples CB1 is localized in the STB cytoplasm, although a slight staining was also observed in the basal membrane of the STB. This observation suggests that labor modulates CB1 localization and thus it may impact its response to AEA stimulation.

We therefore analyzed if CB1 is associated to LRs and if it depends on whether the placenta received labor modulating signals. Our DRMs study showed a translocation of CB1 from the apical membrane of the STB to the cytoplasm in CS placentas, and to the basal membrane in VD samples, where CB1 associates to LRs/caveolae as evidenced by its co-localization with FLOTILIN-1 and CAV-1. Our evidence is in line with the fact that LRs can regulate CB1 signaling by controlling its internalization, and of particular interest is the observation by Keren and Sarne (2003) who described that CB1 internalization may be induced by ligand binding. Therefore, we propose that labor modulating molecules, like AEA, may promote CB1 association to LRs/caveolae and thus internalization into the cytoplasm and translocation to the basal membrane of the STB. Whether this is a mechanism for attenuating CB1 signal requires further studies. However, it has been described that CB1 desensitization is a mechanism dependent on LRs/caveolae, in which CB1 is removed from the membrane via endocytosis (discussed in Dainese et al., 2007). In line with this, our observation on the dual effect of AEA on NOS activity could be related to CB1 residing in LRs and thus its lower availability to the ligand. In our study we observed that in non-laboring CS placentas, CB1 mediates AEA effect since incubation with AM251 prevented NOS activity up-regulation, in line with CB1 localizing in the membrane, out of LRs. In contrast, in VD placentas the inhibitory effect of AEA on NOS activity is not mediated by CB1, concomitant with the notion of LRs/caveolae internalizing CB1. It is plausible that VD placentas, exposed to a broad set of factors that regulate labor, may internalize CB1 as a mechanism for attenuating its signaling. The inhibitory effect of AEA on NOS activity could be exerted by a mechanism independent of receptor that involves the participation of LRs, as it was described in other cell types (Biswas et al., 2003; Siegmund et al., 2005; DeMorrow et al., 2007). This could be a possible mechanism of action of AEA in VD placentas.

In summary, we studied the effect of AEA on placentas before and after the onset of labor and we demonstrated that prior to the onset of labor, AEA is relevant for maintaining elevated placental NOS activity and low PGs concentration, contributing to a quiescent state. On the other hand, changes in the expression of placental ES occur with labor. This could result in a decrease of NO production along with an increase in placental PGs concentration, contributing to the contractile signals that stimulate labor. We postulate that CB1 trafficking plays an important role in the labor process, modulating AEA action. Future experiments are required to elucidate the mechanism underlying CB1 trafficking.

Anandamide's dual action seems to depend on the molecular context of the placenta, in terms of the different scenario stablished by the molecules that regulate labor. We propose that AEA acts as a modulator in the placenta, contributing to the set of signals that participate in the onset of labor at term.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human samples were reviewed and approved by the Ethics Committee of the Center for Medical Education and Clinical Research "Norberto Quirno" (No. 684). All patients provided their written informed consent.

AUTHOR CONTRIBUTIONS

PA and CA performed experiments and wrote the manuscript. TE analyzed the data. MN and GL provided the placentas and the clinical characteristics of the patients. VH and SM performed DRM's technique. MF conceived the study. All authors contributed to the article and approved the submitted version.

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High Levels of Tumor Necrosis Factor-Alpha Reduce Placental Aquaporin 3 Expression and Impair *in vitro* Trophoblastic Cell Migration

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Placentas from preeclamptic women display augmented tumor necrosis factor-alpha (TNF-α) levels with reduced expression of aquaporin 3 (AQP3). However, whether TNF-α modulates AQP3 expression remains to be elucidated. We hypothesize that elevated levels of TNF-α reduce AQP3 expression and negatively impact trophoblastic cell migration. Spontaneously hypertensive rats (SHRs) and Wistar rats (14–16 weeks) were divided into hypertensive and normotensive groups, respectively. Systolic blood pressure (SBP) was measured, and animals mated. In a third group, pregnant SHRs were treated with a TNF-α antagonist, etanercept (0.8 mg/kg, subcutaneously) on days 0, 6, 12, and 18 of pregnancy. Placentas were collected on the 20th day of pregnancy. Human placental explants, from normotensive pregnancies, were incubated with TNF-α (5, 10, and 20 ng/ml) and/or etanercept (1 μg/ml). Swan 71 cells were incubated with TNF-α (10 ng/ml) and/or etanercept (1 μg/ml) and subjected to the wound healing assay. AQP3 expression was assessed by Western blot and TNF-α levels by ELISA. SBP (mmHg) was elevated in the hypertensive group, and etanercept treatment reduced this parameter. Placental TNF-α levels (pg/ml) were higher in the hypertensive group. AQP3 expression was reduced in the hypertensive group, and etanercept treatment reversed this parameter. Explants submitted to TNF-α exposition displayed reduced expression of AQP3, and etanercept incubation reversed it. Trophoblastic cells incubated with TNF-α showed decreased cell migration and reduced AQP3 expression, and etanercept incubation ameliorated it. Altogether, these data demonstrate that high TNF-α levels negatively modulate AQP3 in placental tissue, impairing cell migration, and its relationship in a pregnancy affected by hypertension.

Keywords: placenta, hypertensive pregnancy, preeclampsia, trophoblastic cell, migration

INTRODUCTION

Hypertensive pregnancy is a term commonly used to describe a broad spectrum of conditions, where patients present mild or severe elevations in blood pressure, along with multiple organ dysfunctions (2019). Hypertensive disorders represent the most common complications during pregnancy, ranging around 5–10% of incidence, and are the primary cause of maternal–perinatal mortality and morbidity worldwide (Tooher et al., 2017; Wilkerson and Ogunbodede, 2019). Regarding the hypertensive disorders affecting pregnancies, the most frequent are chronic hypertension, gestational hypertension, preeclampsia (PE) or eclampsia, and chronic hypertension with superimposed PE (Sutton et al., 2018).

The most cited hypothesis about the pathogenesis of hypertensive pregnancies involves reduced placental perfusion in consequence of the inadequate trophoblastic invasion of the myometrium, resulting in poor placental blood supply (hypoperfusion) and diffuse maternal endothelial dysfunction (Braunthal and Brateanu, 2019). However, it is now accepted that a failure in the trophoblast differentiation may lead to hypertensive disorders (Barrientos et al., 2017). Although its etiology remains unclear (Vest and Cho, 2014), there is a consensus that defects in placentation are among the main predisposing factors for PE (Huppertz, 2008; Hawfield and Freedman, 2009).

During placentation, trophoblast cells can differentiate into two different lineages: the villous trophoblast (VT) and the extravillous trophoblast (EVT). While the VT cells cover the chorionic villus and form a syncytium, acting in the fetal–maternal transport, the EVT cells differentiate into interstitial trophoblasts, which can migrate and invade the decidua and the myometrium to transform the maternal spiral arteries. As a result, the spiral arteries acquire the physiological properties necessary to establish adequate maternal blood flow to the developing uteroplacental unit (Davies et al., 2016; Silva and Serakides, 2016).

Tumor necrosis factor-alpha (TNF-α), a pleiotropic cytokine that plays a central role in regulating inflammation, is involved in the pathogenesis of PE, where TNF-α levels are found to be raised in both serum and placental tissue (Weel et al., 2016). Indeed, augmented TNF-α levels during pregnancy are associated with an increased risk for PE development (Zak and Soucek, 2019). Interestingly, TNF-α inhibits trophoblast integration into endothelial cellular networks (Xu et al., 2011) and suppresses the invasion of human trophoblast cells (Wen et al., 2018). However, the exact mechanism behind the TNF-α-mediated reduction of trophoblast cell invasion remains poorly understood.

Lately, the importance of aquaporins (AQPs) during placentation and its importance in fetal development have been recognized (Martinez and Damiano, 2017). Classically, AQPs are water channels that allow the rapid movement of water through the membrane to help maintain homeostasis, also involved in the transport of glycerol (Herrera and Garvin, 2011; Yester and Kuhn, 2017; Delgado-Bermudez et al., 2019). Recently, non-canonical functions of AQPs have also been reported, including proliferation, apoptosis, and cell migration. All these functions are related to temporary changes in cell volume (Kitchen et al., 2015). Remarkably, the expression of AQP3 in PE placentas is reduced (Szpilbarg and Damiano, 2017), and the silencing of AQP3 impairs EVT cell migration (Alejandra et al., 2018) and produces a failure in EVT endovascular differentiation (Reppetti et al., 2020). However, the signal triggering AQP3 reduction during PE is currently unknown.

To address this question, we hypothesized that high TNF-α levels impair cell migration by reducing the expression of AQP3 in placental tissue. In the present study, we investigate whether TNF-α modulates AQP3 in the placenta using a hypertensive pregnancy animal model, as well as an *in vitro* model of human placental explants. Furthermore, we investigate the impact of TNF-α on AQP3 expression and cellular migration in immortalized trophoblast cells from the human placenta.

METHODS

Animals

All procedures were performed following the Guiding Principles in the Care and Use of Animals, adopted by the Brazilian College of Animal Experimentation. The study was approved by the Committee of Ethics in Animal from the Federal University of Mato Grosso (CEUA #23108.038471/2019-14)

Female Wistar rats and spontaneously hypertensive rats (SHRs) (14–16 weeks old, 180–200 g) were used. The rats were maintained in the animal facility room, at 23 ± 2°C, with 12-h light/dark cycles, fed a standard commercial diet, and free water intake. Blood pressure (BP) was measured by tail-cuff plethysmography after 3 days of acclimatization. To mate, females were housed with Wistar males. Vaginal smears were taken daily, and the day on which spermatozoa were found in the vaginal smear was designated gestational day 0.

Experimental Design

Pregnant SHRs and Wistar rats were separated into hypertensive ($n = 6$) and normotensive group ($n = 6$), respectively. The third group was composed of pregnant SHRs ($n = 8$), treated with 0.8 mg/kg etanercept (Embrel®), on days 0, 6, 12, and 18 of pregnancy (DOP) *via* subcutaneous injection as performed previously (Small et al., 2016). On the 20th gestational day, rats were anesthetized with 3% sodium pentobarbital (50 mg/kg body weight, intraperitoneally). After laparotomy for removal of placentas, rats were killed by pneumothorax. The fetuses were individually weighed and classified according to the mean values of fetal weights of the normotensive group as small for gestational age [(SGA) fetal weight < Wistar mean - SD × 1.7]; appropriate for gestational age [(AGA) fetal weight within Wistar mean ± SD × 1.7]; and large for gestational age [(LGA) fetal weight > Wistar mean +SD × 1.7] (Damasceno et al., 2013). Fetuses were killed by placement in a CO_2 chamber.

Tumor Necrosis Factor-Alpha Measurement

Quantitation of TNF-α from placental tissue was performed by ELISA technique using a commercial kit (TNF-α #558535

TABLE 1 | Clinical characteristics of normal pregnant women.

Clinical characteristics	Mean ± SEM
Number of pregnant women	7
Parity	
Primiparous	4
Multiparous	3
Maternal age, years	28.6 ± 2
Gestational age, weeks	38.7 ± 0.4
Mean blood pressure, mmHg	
Systolic	114.3 ± 2
Diastolic	70 ± 2.2
Proteinuria	Negative
Body mass index (BMI), kg/m^2	25.4 ± 1.4
Birth weight, g	3234 ± 147
Fetal sex	
Male	4
Female	3

Values are mean ± SEM.

BD Biosciences, Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Human Tissue Collection

This study was approved by the Human Ethics Committee from the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires (EXP-FYB #0045449/2017) and the Ethics Committee from the *Hospital Nacional Dr. Prof. Alejandro Posadas*, Buenos Aires, Argentina. The investigation conforms to the principles outlined in the Declaration of Helsinki. The patients signed written consent forms before sample collection, and clinical data were collected.

Normal human term placenta ($n = 7$) were obtained immediately after vaginal or cesarean delivery (clinical data are shown in **Table 1**), packed in containers containing ice, and transported to the Reproduction Physiology Laboratory at the University of Buenos Aires.

Explant Culture

Placental cotyledons were gently separated and washed in 0.9% sodium chloride solution to remove blood from the intervillous spaces. Villous tissue was minced with scalpel blades to obtain explants in the proportion of ∼0.5 × 0.5 cm. Explants were cultured in Dulbecco modified Eagle medium (DMEM; Life Technologies, Inc.)/F12 containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 mg/ml streptomycin, and 32 mg/ml gentamicin at 37°C in a humidified gas mixture of 5% CO_2 and 95% air.

Explants were placed in a 24-well plate in the proportion of five explants per well. These were then incubated with complete culture medium containing TNF-α at concentrations of 5, 10, or 20 ng/ml, etanercept at a concentration of 1 μg/ml or vehicle (culture medium) for 16 h.

Explant Viability

Explant viability was verified by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. One explant from each well was removed and transferred to new wells and incubated with 0.5 mg/ml MTT solution (Sigma-Aldrich Corp.) at 37°C for 2 h. Each explant was transferred to a new well, and 100 μl of ethanol was added and incubated for 30 min with shaking at room temperature. Absorbance was measured at 595 nm wavelength.

Explants' toxicity was verified by the release of the intracellular enzyme lactate dehydrogenase (LDH) into the incubation medium after 16 h. Aliquots of the culture medium (500 μl) from each explant were collected and centrifuged. The supernatant was taken (50 μl) and transferred to new wells where they were incubated with a lactate dehydrogenase (LDH) assay solution at room temperature for 30 min. A stop solution was added, and absorbance was measured at 590 nm wavelength.

Cell Culture

Immortalized human trophoblastic cell line Swan 71, described by Straszewski-Chavez (Straszewski-Chavez et al., 2009), obtained from the 7-week placental cytotrophoblast in the first trimester of a healthy pregnancy, was used. Cells were grown in complete culture medium at 37°C for 24 h in a humidified gas mixture of 5% CO_2 and 95% air. At confluence, cells were arrested in the corresponding medium supplemented with 0.5% FBS and incubated with 10 ng/ml TNF-α. Cell viability was assessed by the MTT assay as described previously (Alejandra et al., 2018).

Western Blot Analysis

The total cell lysate was obtained by incubating macerated placenta tissue with lysis buffer containing protease inhibitors, and protein concentration was determined using the Bradford Assay Kit (Sigma-Aldrich). Proteins (50–60 μg) were loaded and separated by electrophoresis on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Sigma-Aldrich). Non-specific binding sites were blocked with 5% skim dry milk in Tris-buffered saline solution with Tween-20 (TBS-T, pH 7.6) for 1 h at room temperature. Membranes were rinsed and incubated with an anti-AQP3 antibody (1:1,000; Alpha Diagnostic Intl. Inc.) followed by incubation with a peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA).

Protein bands were detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and then quantified using an image analysis software program (Kodak Digital Science 1D Image Analysis Software, Eastman Kodak, Rochester, NY, USA).

Wound Healing Assay

Swan 71 cells were cultured in a 24-well plate in complete culture medium at 37°C for 24 h in a humidified gas mixture of 5% CO_2 and 95% air. Once 90% of cell confluence was observed in the plate wells, a wound was made in each cell monolayer, across the center of the wells, using a 200-μl pipette tip. Then, cells were arrested in the corresponding medium supplemented with 0.5%

TABLE 2 | Fetal parameters from Wistar rats and SHRs.

	Wistar rat	SHR
Fetal weight (g)	4.9 ± 0.1	3.4 ± 0.04*
SGA (%)	1.5	100.0*
AGA (%)	97.0	0.0*
LGA (%)	1.5	0.0

*SHR, spontaneously hypertensive rats; SGA, small for gestational age; AGA, appropriate for gestational age; LGA, large for gestational age. *p < 0.05 vs. Wistar. Student's unpaired t-test and Fisher exact test (%).*

FBS and incubated with 10 ng/ml TNF-α, 1 μg/ml etanercept, or vehicle (culture medium).

Wounded monolayers were examined at 0, 18, and 24 h and photographed using an optical microscope with a camera attached. Wound closure was measured by calculating the area (mm^2) using an image analysis software and presented by percentage. The scratch distance at 0 h was considered 0% of gap closure. For migration speed, we measured the width of the wound as the mean distance between the edges of the scratch in each photograph, and this change in mean wound width was divided by the time spent on each migration.

Statistical Analysis

Statistical analysis was performed, and the data were presented as mean ± standard error of the mean (SEM). The significance of the results was analyzed by Student's *t*-test, one-way ANOVA followed by the Tukey posttest or two-way ANOVA followed by the Bonferroni posttest. For the proportion data, the significance was analyzed by Fisher exact test. $p < 0.05$ was considered statistically significant for all experiments.

RESULTS

Fetal Parameters in Spontaneously Hypertensive Rats and Wistar Rats

We assessed the fetal parameters from hypertensive and normotensive pregnant rats. Fetuses from SHRs presented decreased fetal weight (3.4 ± 0.04 g vs. 4.9 ± 0.1 g; $p < 0.0001$) and higher proportion of fetuses classified as SGA (100 vs. 1.5%; $p = 0.000$) compared to Wistar rats (**Table 2**), indicating impairment of fetal development during the hypertensive pregnancy.

Placental Tumor Necrosis Factor-Alpha Levels and Blood Pressure in Spontaneously Hypertensive Rats and Wistar Rats

Given the importance of the placenta in fetal nutrition, and considering that hypertension promotes low-grade inflammation, we analyzed placental TNF-α levels and confirmed the hypertensive pregnancy state by tail-cuff plethysmography. Furthermore, we treated a group of SHRs with etanercept, a TNF-α antagonist. Placental TNF-α levels were greater (3 ± 0.1 pg/ml vs. 1.5 ± 0.3 pg/ml; $p = 0.003$) and systolic blood pressure (SBP) was higher (181 ± 3 mmHg vs. 128 ± 5 mmHg; $p < 0.0001$) in SHRs compared to Wistar rats. Interestingly, when we treated SHRs with etanercept, the SBP was found to be reduced but not normalized [163 ± 2 mmHg; $p = 0.003$ (**Figures 1A,B**)].

Aquaporin 3 Expression in Placental Tissue From Spontaneously Hypertensive Rats and Wistar Rats

To explore the role of TNF-α in AQP3 modulation in the placenta, we performed Western blot analysis of AQP3 in Wistar rats, SHRs, and SHRs treated with etanercept. Placentas from SHRs presented reduced AQP3 expression compared to Wistar rats. Interestingly, etanercept treatment elevated placental AQP3 expression (**Figure 2**). From here, we decided to investigate whether TNF-α levels were related to AQP3 in placental tissue using an *in vitro* model of human placenta explants.

Aquaporin 3 Expression Upon Tumor Necrosis Factor-Alpha Incubation in Normal Human Placenta Explants

Healthy human placental explants were incubated with different concentrations of TNF-α (5, 10, and 20 ng/ml) to evaluate AQP3 expression. Treatment with TNF-α did not affect explants' viability or toxicity (**Figures 3A,B**). TNF-α reduced the expression of AQP3 upon 10 and 20 ng/ml TNF-α incubation (**Figure 3C**). Additionally, simultaneous incubation with 10 ng/ml TNF-α and 1 μg/ml etanercept increased AQP3 expression (**Figure 3D**).

Aquaporin 3 Expression and Cellular Migration in Extravillous Trophoblast Cells Incubated With Tumor Necrosis Factor-Alpha

To investigate the role of TNF-α in EVT cell migration, Swan 71 cells were incubated with TNF-α and/or etanercept. AQP3 expression in Swan 71 cells was reduced after 10 ng/ml TNF-α incubation (**Figures 4A,B**). Additionally, *in vitro* experiments demonstrated that incubation with 10 ng/ml TNF-α impaired wound healing (**Figures 4D–F**). Concomitant incubation with TNF-α and 1 μg/ml etanercept improved the wound repair process. Moreover, incubation with TNF-α significantly decreased Swan 71 cell migration speed, and concomitant incubation with TNF-α and etanercept raised the migration rate (**Figure 4E**). Treatment with TNF-α did not affect cellular viability (**Figure 4C**).

DISCUSSION

The present study shows that hypertensive rats present decreased fetal weight and increased proportion of SGA fetuses concomitant with augmented placental levels of TNF-α and reduced placental AQP3 expression when compared to the normotensive group. Interestingly, etanercept treatment was able to both reduce BP levels and restore placental AQP3 expression in SHRs. The SHR animal model naturally shows an increase in BP after the 10th week of life (Breckenridge, 2013; Thoonen et al.,

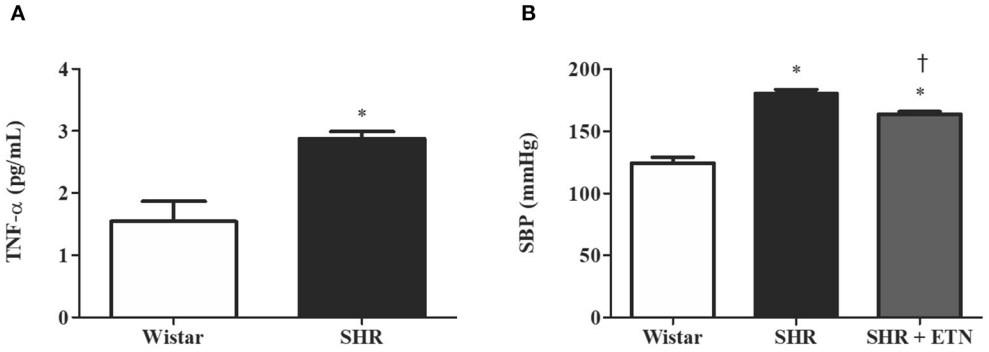

FIGURE 1 | Placental tumor necrosis factor-alpha (TNF-α) levels and systolic blood pressure (SBP) are greater in spontaneously hypertensive rats (SHRs) compared to Wistar rats, and etanercept treatment reduces, without normalizing, SBP in treated SHRs. **(A)** Bar graph showing placental TNF-α levels (pg/ml) in SHR and Wistar; $n = 6$. **(B)** Bar graph showing SBP (mmHg) in SHR, Wistar, and SHR treated with etanercept; $n = 5$. Values are presented as means ± SEM, and data were analyzed by **(A)** Student's *t*-test or **(B)** one-way ANOVA, followed by Tukey posttest. $^{*}p < 0.05$ vs. Wistar; $^{\dagger}p < 0.05$ vs. SHR.

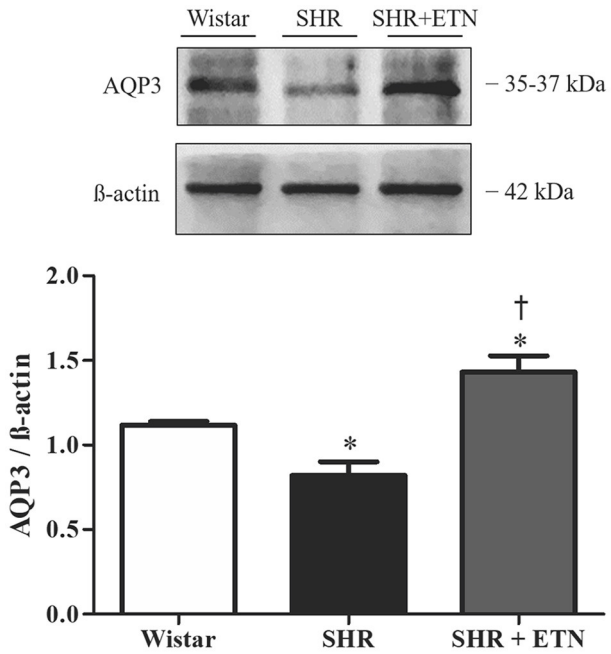

FIGURE 2 | Placental aquaporin 3 (AQP3) expression is decreased in spontaneously hypertensive rats (SHRs) compared to Wistar rats, and etanercept treatment ameliorates SHR AQP3 expression. Upper representative picture of Western blot membrane with the respective AQP3 molecular weight. Bar graph showing the AQP3 expression in placentas from SHR, Wistar, and SHR treated with etanercept (ETN) after normalization to β-actin expression; $n = 5$. Values are presented as means ± SEM, and data were analyzed by one-way ANOVA, followed by Tukey posttest. $^{*}p < 0.05$ vs. Wistar; $^{\dagger}p < 0.05$ vs. SHR.

2015) mainly due to genetic predisposition to higher sodium. However, other mechanisms of BP increase have been proposed for SHRs, including a pro-inflammatory environment. During hypertension, a low-grade inflammatory condition is established with the secretion of inflammatory cytokines, including TNF-α (Mehaffey and Majid, 2017). This cytokine is majorly secreted by Th1 lymphocytes, and TNF-α is frequently involved in inflammatory and immunological processes occurring during arterial hypertension (Barbaro et al., 2015). Etanercept is a soluble recombinant TNF receptor that acts by inhibiting TNF-α action (Guillot et al., 2017). The reduced BP observed in SHRs treated with etanercept indicates that TNF-α plays a role in the maintenance of BP elevation, supporting the idea that TNF-α is part of the pathophysiology of high BP (Mehaffey and Majid, 2017; Lu and Crowley, 2018). Moreover, high levels of TNF-α are related to numerous obstetric complications, including recurrent abortions, failures in the implantation process, and PE (Saito et al., 2010).

AQPs are water channels playing a significant role in maintaining hydric homeostasis, allowing the rapid movement of water through the membranes (Roche and Tornroth-Horsefield, 2017). In this regard, AQPs play many roles in controlling cellular volume by being permeable to water but also, in some cases, to glycerol and urea (Ducza et al., 2017). Lately, AQPs have gained further interest since other features have been attributed to these water channels, including proliferation, apoptosis, and cell migration (Kitchen et al., 2015). In the placenta, AQPs are mainly expressed in the apical membrane of syncytiotrophoblast cells (Damiano et al., 2001). Although the exact function of AQPs in trophoblast cells remains unclear, some authors have recently shown that AQP3 blockade impairs EVT cell migration (Alejandra et al., 2018). Once AQP3 expression in SHR placenta is reduced, and considering that TNF-α inhibition ameliorated AQP3 placental expression, we investigated whether complications observed in hypertensive pregnancies may be due to AQP3. This hypothesis is further supported by the fact that AQP3 expression is reduced in placentas from human pregnancies complicated by PE (Szpilbarg and Damiano, 2017).

The inverse relation between TNF-α and AQP3 expression obtained in SHR placentas was further confirmed in human placentas. An *in vitro* study was performed by incubating normotensive human placental explants with TNF-α. Here, we

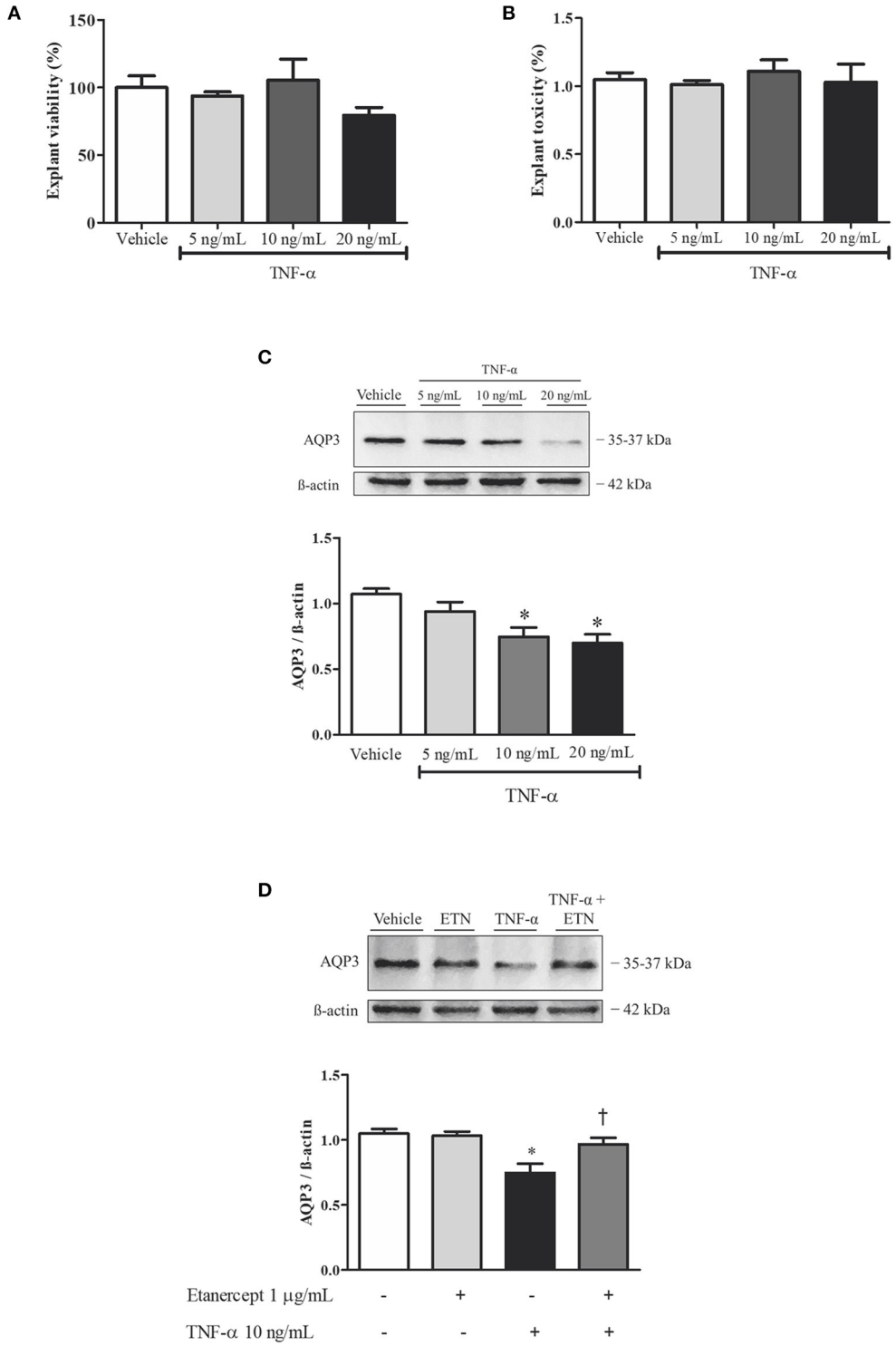

FIGURE 3 | Tumor necrosis factor-alpha (TNF-α) incubation reduces aquaporin 3 (AQP3) expression in normal human placenta explants, and simultaneous incubation with TNF-α and etanercept (ETN) retrieves AQP3 expression. **(A,B)** Bar graphs showing explant viability and toxicity, respectively; $n = 3$. **(C)** Upper representative picture of Western blot membrane with the respective AQP3 molecular weight. Bar graphs showing the AQP3 expression upon incubation with vehicle ($n = 7$), 5 ng/ml TNF-α ($n = 6$), 10 ng/ml TNF-α ($n = 6$), and 20 ng/ml TNF-α ($n = 7$). **(D)** Bar graphs showing the AQP3 expression upon incubation with vehicle ($n = 7$), 1 µg/ml etanercept ($n = 6$), 10 ng/ml TNF-α ($n = 6$), and simultaneous TNF-α and etanercept ($n = 6$). Values are presented as means ± SEM, and data were analyzed by one-way ANOVA, followed by Tukey posttest. $^{*}p < 0.05$ vs. vehicle; $^{\dagger}p < 0.05$ vs. 10 ng/ml TNF-α incubation.

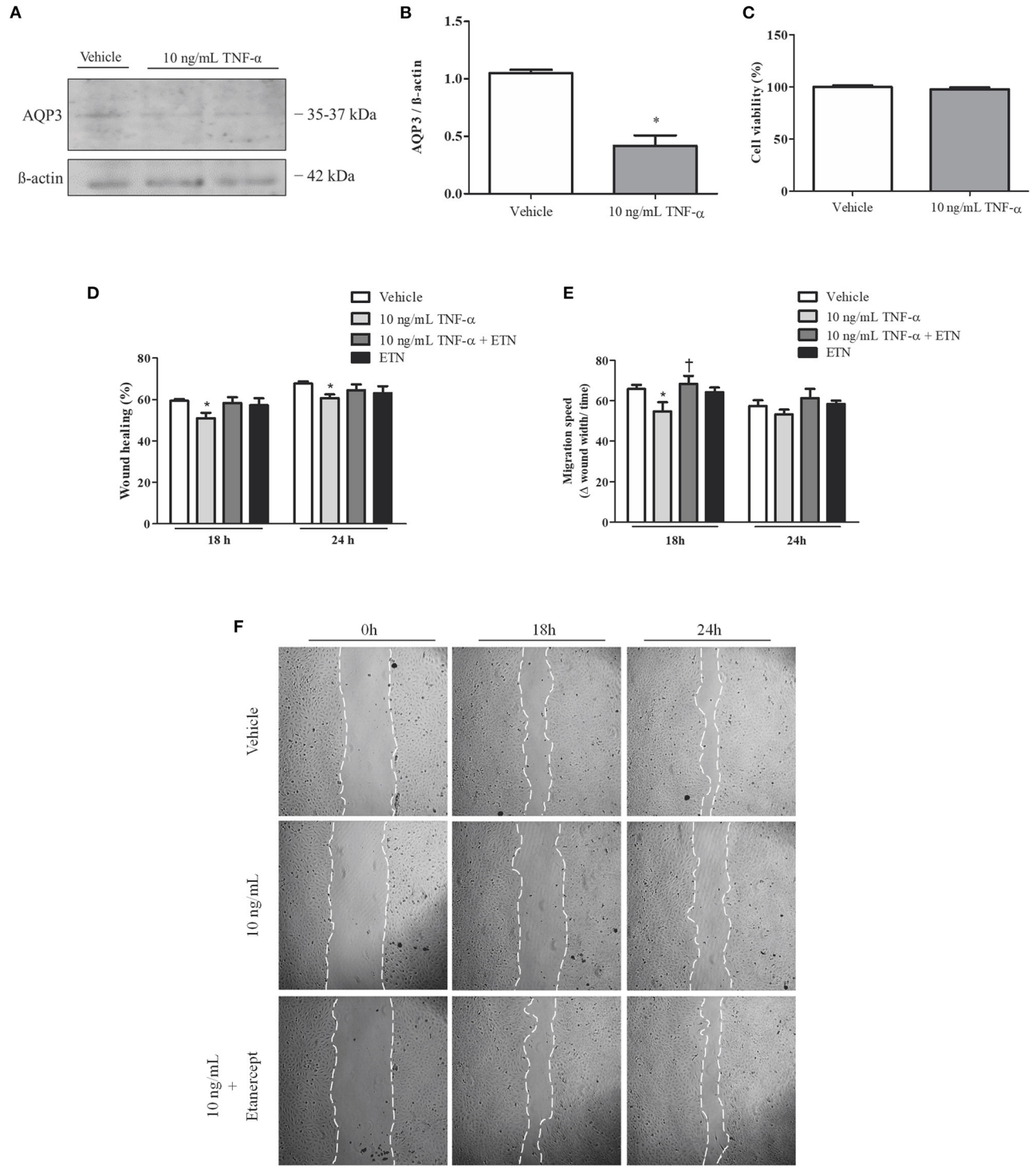

FIGURE 4 | Tumor necrosis factor-alpha (TNF-α) reduces aquaporin 3 (AQP3) expression and impairs human trophoblastic cell migration. Concomitantly, simultaneous incubation with TNF-α and etanercept (ETN) ameliorates cell migration speed. **(A)** Representative picture of Western blot membrane with the respective AQP3 molecular weight. **(B)** Bar graph showing AQP3 expression in Swan 71 cells incubated with vehicle ($n = 5$) or 10 ng/ml TNF-α ($n = 6$). **(C)** Bar graph showing cellular viability ($n = 5$). **(D)** Bar graph showing the wound healing percentage in Swan 71 cells treated with vehicle ($n = 6$), 10 ng/ml TNF-α ($n = 7$), TNF-α + etanercept ($n = 3$), or 1 μg/ml etanercept ($n = 2$) after 18 and 24 h. **(E)** Bar graph showing the migration speed in Swan 71 cells treated with vehicle, 10 ng/ml TNF-α, TNF-α + etanercept, or 1 μg/ml etanercept after 18 and 24 h; $n = 4$. **(F)** Representative pictures of wound healing assay of Swan 71 cells incubated with or without 10 ng/ml TNF-α and 1 μg/ml etanercept after 0, 18, and 24 h. Values are presented as means ± SEM, and data were analyzed by two-way ANOVA, followed by Bonferroni posttest. $^{*}p < 0.05$ vs. vehicle; $^{\dagger}p < 0.05$ vs. 18 h 10 ng/ml TNF-α incubation.

aimed to test a concentration-dependent response using TNF-α at concentrations of 5, 10, or 20 ng/ml. Interestingly, TNF-α incubation negatively modulates AQP3 expression in placental explants, where both 10 and 20 ng/ml of TNF-α significantly reduced AQP3 expression. These data were confirmed when the explants were concomitantly incubated with TNF-α and 1 μg/ml etanercept, and the AQP3 expression was retrieved. Additionally, a previous study showed that TNF-α promoted a downregulation of AQP3 expression in human colonic adenocarcinoma (HT-29) cells through inhibition of constitutive transcriptional activity of the AQP3 promoter (Peplowski et al., 2017). Although 10 ng/ml TNF-α is a higher concentration than that observed in *in vivo* levels, it is in accordance with other studies using placental explants and is the most used concentration (Bauer et al., 2004; Leisser et al., 2006; Siwetz et al., 2016), providing the concentration range for TNF-α used in this study. Moreover, etanercept (1 μg/ml) incubation was used to inhibit the TNF-α actions before, as previously published by our group (Castro Parodi et al., 2011).

Human placental explants are constituted of placental villi, mainly composed of a specific trophoblastic cell type called cytotrophoblasts (CTBs). The arrangement of CTBs forms multinucleated syncytiotrophoblasts. During pregnancy progression, the CTB differentiates into the EVT, a cell type with migration properties that can invade the uterine wall. The reduced AQP3 expression observed in placental explants indicates that this event is happening before trophoblast differentiation into VT and EVT, probably being related to the most severe cases of PE, known as early-onset PE (Raymond and Peterson, 2011). This is the most critical subtype of PE, as it is associated with fetal growth restriction and is the major cause of PE-related morbidities and mortalities of mother and child (Huppertz, 2018). Moreover, the fact that hypertensive animals present both reduced fetal weight and AQP3 expression leads us to believe that deficient AQP3 is involved in fetal growth restriction. Interestingly, a previous study found that AQP3 null mice presented smaller fetuses, suggesting a role for AQP3 in fetal growth (Seo et al., 2018).

Although the complete mechanism eliciting PE remains unknown, its pathophysiology involves abnormal placentation and inadequate development of the uterine–placental vascular system (Williamson et al., 2017). Uteroplacental blood flow is allowed by a correct EVT invasion in uterine spiral arteries; however, prior to invasion into arteries, interstitial trophoblasts migrate deep into the uterus and reach the myometrium (Moser et al., 2018). This migration process is critical for EVT interaction with arteries and, consequently, for uteroplacental blood flow development (Pollheimer et al., 2018). In the present study, *in vitro*, TNF-α incubation impaired both EVT cell wound healing process and migration speed and reduced AQP3 expression in these cells. Concomitantly, simultaneous incubation with TNF-α and etanercept ameliorated EVT migration speed in these cells. These data indicate that TNF-α plays a deleterious role in migration by reducing AQP3 expression.

Besides TNF-α, the influence of other cytokines, especially the interleukins (ILs), on trophoblastic cell migration has been under study. IL-6, IL-8, IL-11, and IL-1β were found to stimulate *in vitro* trophoblast cell migration (Paiva et al., 2007; Hirota et al., 2009; Jovanovic and Vicovac, 2009; Jovanovic et al., 2010), while IL-27 incubation inhibited trophoblast cell migration and invasion (Ge et al., 2019). However, the relationship between these cytokines and AQP3 has not been established yet and must be addressed in future studies.

Evidence shows that AQPs may be involved in tumor cell migration (Cao et al., 2013; Ribatti et al., 2014). In cultured cancer cells, increased AQP3 expression increases cell proliferation, migration, and invasion, playing a pivotal and complex role in cancer progression (Marlar et al., 2017). Trophoblast cells of the human placenta use a very similar machinery for the cancer cells to grow, migrate, and invade but in a tightly regulated way (Soundararajan and Rao, 2004). In human placental trophoblastic cells, AQP3 inhibition or silenced AQP3 gene expression has reduced trophoblastic migration (Alejandra et al., 2018). Moreover, reduced AQP3 expression also reduces trophoblast endovascular differentiation and affects tubule formation (Reppetti et al., 2020). Our findings strongly support the hypothesis that TNF-α negatively impacts trophoblastic cell migration by reducing AQP3 expression. This observation is supported by the fact that TNF-α can impair *in vitro* trophoblast migration (Bauer et al., 2004).

In conclusion, the present study demonstrates, for the first time, the harmful modulation of AQP3 by augmented TNF-α levels in placental tissue, affecting trophoblast cell migration. This relationship is also observed in placental tissue from hypertensive pregnancy. Altogether, these observations contribute to a better understanding of placental impairment during hypertensive disorders of pregnancy.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Ethics Committee from the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Committee of Ethics in Animal from the Federal University of Mato Grosso.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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BRIEF RESEARCH REPORT
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Assessment of Placental Extracellular Vesicles-Associated Fas Ligand and TNF-Related Apoptosis-Inducing Ligand in Pregnancies Complicated by Early and Late Onset Preeclampsia

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Preeclampsia (PE) is a hypertensive disorder that affects 2–8% of pregnancies and is one of the main causes of fetal, neonatal, and maternal mortality and morbidity worldwide. Although PE etiology and pathophysiology remain unknown, there is evidence that the hyperactivation of maternal immunity cells against placental cells triggers trophoblast cell apoptosis and death. It has also been reported that placenta-derived extracellular vesicles (EV) carry Fas ligand (FasL) and Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and trigger apoptosis in Jurkat T cells. This study aimed to quantify and compare FasL and TRAIL expression in EV derived from cultures of placenta explants from women with PE (early versus late) and women with uncomplicated pregnancies. Also, the study assessed EV capacity to induce apoptosis in Jurkat T cells. The authors isolated EV from placenta explant cultures, quantified FasL and TRAIL using ELISA, and analyzed EV apoptosis-inducing capability by flow cytometry. Results showed increased FasL and TRAIL in EV derived from placenta of women with PE, and increased EV apoptosis-inducing capability in Jurkat T cells. These results offer supporting evidence that EV FasL and TRAIL play a role in the pathophysiology of PE.

Keywords: pregnancy, hypertension disorder complicating pregnancy, exosome (vesicle), apoptosis, placental culture

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INTRODUCTION

Preeclampsia (PE) is a hypertensive disorder of pregnancy with an impact on perinatal and maternal mortality and morbidity. According to the World Health Organization (WHO), maternal mortality due to such hypertensive disorders is 2.8% of pregnancies worldwide (Say et al., 2014). The mortality rate for PE in Latin America and the Caribbean is 26% (Steegers et al., 2010), and in

Colombia 20–30% (Khan et al., 2006). Also, one fourth of fetal and neonatal deceases worldwide is associated with PE (500,000 neonates per year) (Duley, 2009).

This multifactorial disease is of unknown pathophysiology. The evidence, though, suggests the placenta plays an important role in the development of the disease. Increased proliferation and fusion of the cytotrophoblast, abnormal/incomplete remodeling of uterine spiral arteries (UtA), failed trophoblastic invasion, abnormal placentation, placental insufficiency, and an increased release of placenta-derived factors and EV have been described. These EV would have proinflammatory, anti-angiogenic, prothrombotic, and apoptotic properties. It is possible that placental molecules and membrane particles released in maternal circulation play a role in the systemic inflammation, endothelial dysfunction, and multi-systemic involvement characteristic of PE (Tannetta et al., 2017).

Currently, there are two categories of PE: Early-onset PE (EO-PE) (gestation $<$ 34 weeks), and late-onset PE (LO-PE) (gestation $\geq$ 34 weeks). The two sub-types seem to have different etiology, pathophysiology, phenotype, and prognosis (Gilani et al., 2016). The EO-PE apparently involves an abnormal placentation and incomplete/failed remodeling of UtA. This type of PE has a higher risk of maternal and fetal complications such as intrauterine growth restriction (IUGR). The LO-PE possibly arises from the interaction between an apparently normal placenta and a maternal susceptibility involving endothelial dysfunction (microvascular damage) that results in general vasoconstriction and reduction of blood flow to multiple organs (heart, kidney, brain). This type of PE has a lower rate of fetal involvement and fewer perinatal complications than EO-PE (Phipps et al., 2016).

One of the principal mechanisms involved in PE development is apoptosis, associated with reduced/altered syncitiothrofoblast formation, abnormal UtA remodeling, increased proinflammatory cytokines, reduced maternal-fetal immune tolerance, and increased expression of apoptotic proteins such as FasL and TRAIL in placenta-released EV (Sharp et al., 2010). TRAIL is a type II membrane protein. This protein induces apoptosis in transformed cells and tumor cells, is expressed at a significant level in most normal tissues and has been implicated in processes of homeostasis, autoimmune suppression, immune surveillance, among others (Stelzer et al., 2016). FasL is a type II membrane protein that triggers death signals by binding to its membrane receptor Fas (Stenqvist et al., 2013). This protein is involved in the development of organs and in the homeostasis of cells and tissues. It is expressed in activated and cytotoxic T lymphocytes, NK cells, neutrophils, and cells found in immune privileged sites such as the eye, brain, testes, and placenta (Stelzer et al., 2016). It has been reported that FasL and TRAIL are expressed by trophoblast cells, which can induce apoptosis in activated lymphocytes and, therefore, provide a mechanism for the maternal immune privilege of the fetus, in addition to a regulation of placental homeostasis during trophoblastic invasion (Jerzak and Bischof, 2002). Different studies have shown that FasL is involved in the remodeling of the UtA and in the establishment of placental tolerance and immune privilege (Bai et al., 2009), for its part, the participation of TRAIL in the remodeling of UtA, where Cytotrophoblast uses a TRAIL-dependent mechanism to induce smooth muscle cell death and is involved in vessel remodeling (Keogh et al., 2007).

Our hypothesis was: there are differences between the amount of FasL and TRAIL in extracellular vesicles derived from culture of placental explants of pregnant women with uncomplicated pregnancy and pregnant women with diagnosis of EO-PE or LO-PE and these EVs are capable to induce apoptosis. This study aimed to assess the presence of FasL and TRAIL in EV released by the placentas of patients with EO-PE and LO-PE, and the apoptosis-inducing capability of those vesicle-associated proteins.

MATERIALS AND METHODS

Sample Collection

This study was conducted in the Hospital Universitario San Ignacio (HUSI) from February to December, 2018. The samples were placentas of pregnant women with PE after cesarean section delivery (cases, n = 14). The study divided cases in 2 groups of 7 samples each: EO-PE (placentas from women diagnosed with PE that started at $<$34 week gestation) and LO-PE (placentas from women diagnosed with PE that started at $\geq$34 week gestation). Convenience sampling was performed.

Preeclampsia was diagnosed as the American College of Obstetricians and Gynecologists (ACOG) (The American College of Obstetricians and Gynecologists, 2019) recommendations. Placentas of women with uncomplicated pregnancies and healthy newborns were included as controls (controls, n = 7). Women with diabetes, kidney disease, intrapartum infection, or pregnancy complications, such as gestational diabetes, chorioamnionitis, and premature rupture of membranes were excluded. The authors reviewed the clinical histories of candidates for the study to determine whether they fulfilled inclusion criteria. The authors then informed potential participants of the research project and obtained informed consent from those choosing to participate. The placentas taken from C-sections were stored in plastic containers at 4°C in the Pathology laboratory and processed within 12 h of collection. The Ethics Committee of the HUSI-Pontificia Universidad Javeriana Faculty of Medicine approved the project (FM-CIE-0410-17). All procedures were carried out according to the biosafety manual of the Human Genetics Institute and the HUSI.

Human Chorionic Villous Xplants Cultures

Each placental cotyledon was isolated using sterile dissection. The fetal and decidual tissues were removed, and blood from the interstitial space was washed out with 0.9% saline solution. Small fragments (explants) of approximately 0.5 μm were cut out. Two 6-well polyethylene plates were used for each sample. Four explants were incubated for 24 h (Miller et al., 2005) in each well in 8 ml of fetal bovine serum-free DMEM media (Gibco BRL, Bethesda, MD, United States) at 37°C (5% CO_2). Culture viability was assessed using metabolic reduction of 3-(4,5-dimethylthiazol-2-ilo)-2,5-dypheniltetrazole bromide

(MTT) (Sigma-Aldrich, St Louis, MO). Evaluation of culture cell function was by ß-hCG measurement by ELISA (Thermo Fisher Scientific Inc., Waltham, MA, United States).

EV Collection

After 24 h of incubation, the supernatant of explants culture was centrifuged at 4,000 × *g* for 20 min. The pellet was used for another study, and the supernatant was ultra-centrifuged at 100,000 × *g* for 100 min. The supernatant was discarded, and the pellet re-suspended in 8 ml of PBS 1X. After a second ultracentrifugation of the supernatant at 100,000 × *g* for 90 min at 4°C, the supernatant was discarded, and the pellet again re-suspended in 50 μL of PBS 1X. The pellet was stored in 1.5 ml sterile vials at -20°C (Bautista et al., 2015).

EV Quantification and Characterization

Nanoparticles Tracking Analysis (NTA)

For the NTA, a 2 ml pool was performed for EV samples. The diluted samples were loaded into the assembled sample chamber of a NanoSight NS300. The EV were brought into focus using the thumbprint region as a reference, and 60-s video images were acquired and analyzed with NanoSight NTA 3.4 software. The values obtained represent the mean and standard deviation of two replicate isolations.

Western Blot

The authors used Bradford colorimetric method (BIORAD) for total EV protein quantification. First, the standard curve was prepared, and then the samples loaded. EV protein lysates were resolved on Tris-Glycine SDS-PAGE and transferred to polyvinylidene difluoride (BIORAD) membranes. Membranes were incubated overnight in primary antibody CD63 (Invitrogen anti-CD63 catalog #10628D, diluted 1:500 per manufacturer instructions) at 4°C, washed three times with 0.1% TBST, incubated with secondary antibody (HRP-conjugated goat anti-mouse, Thermo Scientific, Pierce, 1:10,000) for 1 h at room temperature, washed three times and detected with enhanced chemiluminescence (Invitrogen) on CL-XPosure Film (Thermo Scientific, Pierce). In addition, IgG1 antibody was used as isotype control (Catalog # 02-6502; Invitrogen).

EV Quantification by ELISA

The ELISA ExoQuant^{TM} Overall Exosome Capture and Quantification Assay Kit (BioVision, California) was used for EV quantification, following the manufacturer's protocol.

VE FasL and TRAIL Quantification

The EV FasL quantification used the ELISA *Fas Ligand (APTL) Human Kit* (Abcam, Cambridge, MA, United States). The EV TRAIL quantification used ELISA *Human TRAIL/TNFSF10 (Tumor Necrosis Factor Related Apoptosis Inducing Ligand) K*it (Elabscience, Houston, Texas, United States). Manufacturer's instructions were followed in both cases.

Apoptosis Induction

The authors cultured 400,000 Jurkat T cells per well in 1 ml of RPMI-supplemented medium in three 12-well polyethylene plates. A 5mg/ml and 10 mg/ml EV protein concentration of 4 EV-sample pool from controls and from a 4 EV-sample pool from cases were added to Jurkat T cells. The samples were incubated at 37°C (5% CO_2) for 8 and 24 h. Each reading assessed negative controls (Jurkat T cells with no EV exposure) and positive controls (Jurkat T cells + 5% DMSO) (Hajighasemi and Tajik, 2017). The FlowTACS^{TM} Apoptosis Detection Kit (Trevigen, Gaithersburg, MD, United States) was used to detect DNA fragmentation due to the apoptotic signaling cascade by flow cytometry in the BD FACSAria^{TM} III sorter (BD Bioscience, San Jose, CA, United States), following the manufacturer's protocol. BDTM CompBeads were used to adjust the fluorescence signals and voltage for each detector. BDTM Cytometer Setup & Tracking Beads (CST) were used for daily evaluation of flow cytometer performance, also following the manufacturer's recommendations.

Statistical Analysis

The authors used the Mann-Whitney statistical test for a comparative analysis of demographic variables among groups. The Fisher exact test was used to compare categorical variables. The Mann-Whitney statistical test was also used to analyze differences in quantification of EV, EV total proteins, and EV FasL and TRAIL expression levels among groups. The tests were assessed with a 95% confidence level. Finally, flow cytometry was analyzed with the *FlowJo* v8 software, and a descriptive analysis was made of apoptosis percentage for each treatment.

RESULTS

There were statistically differences among cases and controls in newborn weight, gestational age at delivery, and blood pressure. Birth weight and gestational age at delivery were lower in cases, especially in EO-PE, compared to controls. The ratio of newborn weight/placenta weight was lower in EO-PE, compared to controls. As expected, gestational age at delivery was higher in LO-PE women compared to EO-PE women. Of the 14 pregnant women with PE, 8 (57.1%) presented PE severity criteria (blood pressure $\geq$ 160 mm Hg/ $\geq$ 110 mm Hg). In addition, 11 (78.5%) PE pregnant women had proteinuria as measured by urine protein/creatinine ratio (**Table 1**).

Results of the nanoparticle tracking analysis allowed determination of EV isolation (Dragovic et al., 2015). The EV had an average size of 149.4 nm with standard deviation of 83.5 nm and concentration of $8.93e^{8} \pm 3.74e^{7}$ particles/ml. Western blot for CD63 showed a ∼55 kDa band indicating the presence of EV enriched with exosomes in the process of isolation (Zhang et al., 2019) (**Figure 1**). The EV quantification showed a lower amount in samples from EO-PE (median: 0.24 ug/ul) compared to controls (median: 4.75 ug/ul) and compared to LO-PE cases (median: 9.36 ug/ul). The total protein quantification showed no statistically significant differences among groups (control group median: 228.5 mg/ml; EO-PE group median: 2,223 mg/ml; LO-PE group median: 268.7 mg/ml). The proportion of total protein/EV was higher in the EO-PE (median: 1,639-fold)

compared to controls (median: 7.88-fold) and compared to the LO-PE group (median: 24.27-fold) (**Figures 1C,D,E**).

Regarding EV FasL expression, concentration in EO-PE (median 178.6 pg/ml) was higher compared to controls (median: 122 pg/ml). No differences were found between EO-PE and LO-PE (median 164.2 pg/ml) (**Figure 2A**). The FasL/EV proportion was higher in the EO-PE (median: 53.2×10^{-5}-fold) compared to controls (median: 3.85×10^{-5}-fold) and compared to the EO-PE group (median: 5.42×10^{-5}-fold) (**Figure 2B**).

Results of EV TRAIL do not show statistically significant differences among groups (median for EO-PE: 4.17 pg/ml; for LO-PE: 3.05 pg/ml; for controls: 3.50 pg/ml) (**Figure 2C**). For the proportion of FasL concentration/EV concentration, the EO-PE (median: 7.57×10^{-6}-fold) value was higher compared to the control group (median: 1.16×10^{-6}-fold) and compared to the LO-PE group (median: 0.90×10^{-6}-fold) (**Figure 2D**).

Conversely, analysis of apoptosis in the EV-treated Jurkat T cells showed a directly proportional relationship between EV concentration increase and apoptosis percentage, at 8 and at 24 h. There was a reduction, however, in the apoptosis percentage at 24 h, both in cases and in controls (**Figure 3** and **Supplementary Figures 1**, **2**).

DISCUSSION

Pregnant women with EO-PE had less favorable obstetric results with high blood pressure levels (severity criterion), premature deliveries (gestation $\leq$ 36 weeks), and low birth weights, compared to controls and to LO-PE pregnant women. This is consistent with previous studies (Lok et al., 2008; Nguyen et al., 2019).

This study shows that placental explants from LO-PE pregnancies have a higher EV release *in vitro*. This phenomenon has been described by Dragovic *et al.*, who reported an increased release of syncytiotrophoblast-derived EV in LO-PE patients and controls compared to those in non-pregnant women (Dragovic et al., 2013). Several authors have found a significant increase of circulating exosomes in EO-PE women, possibly associated with abnormal placental development and reduced function

TABLE 1 | Clinical and demographic characteristics of the population.

Variables	Controls (*n* = 7)		Cases						
			EO-PE (*n* = 7)			LO-PE (*n* = 7)			
	Median	Range	Median	Range	p value	Median	Range	p value	EO-PE vs. LO-PE p value
Maternal age (years)	31	26 – 39	34	23 – 42	0,9283	27	17 – 37	0,1719	0,1731
Gestational age at diagnostic (PE)	N/A	N/A	32,5	24,0 – 33,5	N/A	35,3	34,5 – 38,3	N/A	**0,0012**
Diastolic Blood pressure (mmHg)	62	58 –88	112	76 – 118	**0.008**	92	76 – 131	**0,01**	0,2554
Systolic Blood pressure (mmHg)	123	90 – 130	168,5	158 – 180	**0,001**	164,5	136 – 184	**0,001**	0,6753
Birth Weight (g)	3380	2800 – 3780	2287	1380 – 2590	**0,0006**	2640	2245 – 2950	**0,004**	**0,0175**
Gestational Age (weeks)	39	38 – 40	35	31 –36	**0,0006**	37	34 – 38	**0,004**	**0,0105**
Placental Weight (g)	403,9	328,9 – 439,2	397,7	138,0 – 430,0	0,3759	367,6	301,7 – 443,1	0,5221	0,9656
Birth weight/plcenta ratio (g)	8,7	6,7–9,9	6,42	5,3–10	**0,037**	7,1	6,4–8,0	0,097	0,097
Proteinuria/creatinuria ratio	N/A	N/A	0.9	0,2 – 14,7	N/A	0.55	0,3 – 18,2	N/A	N/A
	N	**%**	**n**	**%**	**p value**	**n**	**%**	**p value**	
Primigravity									
Yes	1	14,3	0	0	1,0	2	28,6	0,6	
No	6	85,7	7	100		5	71,4		
Newborn sex									
Female	2	28,6	4	57,1	0,5921	3	42,9	1,0	
Male	5	71,4	3	42,9		4	57,1		
IUGR									
Yes	0	0	1	14,3	1,0	1	14,3	1,0	
No	7	100	6	85,7		6	85,7		

EO-PE, Early-onset preeclampsia; LO-PE, Late-onset preeclampsia; IUGR, Intrauterine growth restriction. Numbers in bold show p values <0.05.

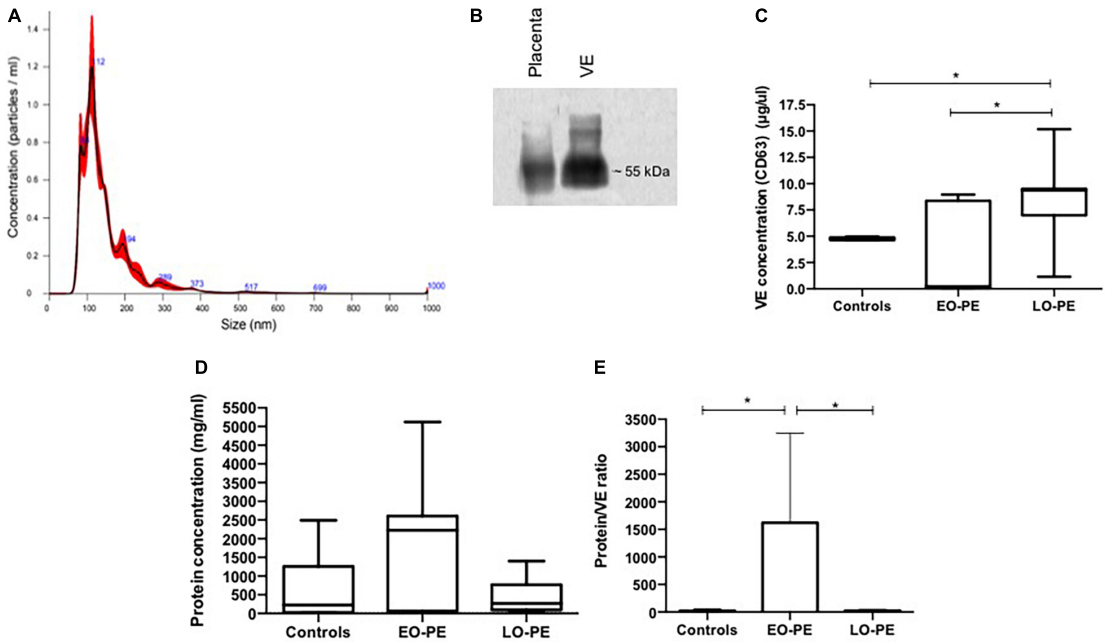

FIGURE 1 | VE characterization from explant culture supernatant. **(A)** Representative vesicle size distribution (nm) by NTA of VE. **(B)** Western blot analysis for the CD63 exosome marker. **(C)** Quantification of VE by ELISA measuring CD63 (controls vs. EO-PE p = 0,2069; controls vs. LO-PE p = 0,0262; EO-PE vs. LO-PE: p = 0,0175). **(D)** Quantification of protein within VE by Bradford method (controls vs. EO-PE p = 0,2069; controls vs. LO-PE p = 0,6031; EO-PE vs. LO-PE: p = 0,3129). **(E)** Analysis of ratio of protein quantity by VE (controls vs. EO-PE p = 0,0175; controls vs. LO-PE p = 0,3759; EO-PE vs. LO-PE: p = 0,0262) *$p \leq 0,05$.

(Lok et al., 2008; Redman et al., 2012; Pillay et al., 2016). Meanwhile, Marques *et al.* did not find significant differences in EV concentrations for PE patients and normotensive pregnant women (Marques et al., 2012). Discrepancy between these results may be due to differences in the types of analyzed samples and the methods for EV isolation and identification (Dragovic et al., 2013). Mitchell *et al.* propose that exosome release may reflect the placental function and metabolic status (Mitchell et al., 2015). It has been reported that in LO-PE the problem arises from interaction between a presumably normal placenta and maternal factors plagued with endothelial dysfunction, making them susceptible to microvascular damage (Phipps et al., 2016). The increased exosome release we report in LO-PE placentas could be interesting and need more research, this finding might be related to a compensation mechanism that attempts to mitigate a pregestational endothelial dysfunction. Further studies are necessary to prove that hypothesis. It is important to note that EV release has been reported to increase with gestational age (Salomon et al., 2014), so differences in EV amounts between EO-PE and LO-PE may be due to gestational age.

By contrast, assessment of the total proteins/EV proportion reveals an increase in the amount of total proteins per EV in EO-PE cases. This finding is consistent with some reports indicating an increased release of EV with altered molecular characteristics (mainly in the bioactive charge) during PE development. These altered characteristics may affect normal EV biological function (Tannetta et al., 2017). The EV express sFlt-1 and endoglin that may contribute to endothelial dysfunction. The EV also express plasminogen-activator inhibitors (PAI-1/PAI-2) responsible for the high levels of fibrin deposition in the intervillous space and for the placental infarctions observed in PE. The PAI are also responsible for excessive oxidation that may amplify the EV inflammatory burden modifying STB proteins and lipids that are not proinflammatory (Tannetta and Sargent, 2013).

Some studies have shown the effect of placental-derived exosomes in the maternal immune modulation during pregnancy, partly through the expression of pro-apoptotic molecules as FasL and TRAIL (Mitchell et al., 2015). FasL may induce the stimulation/activation of the vascular endothelium. FasL may also induce an altered trophoblastic apoptosis that would play a role in endothelial dysfunction, systemic inflammation, and hypertension (Gibbens et al., 2017). Conversely, it has also been reported that FasL may induce apoptosis in activated lymphocytes as a mechanism of tolerance and immunological privilege (Jerzak and Bischof, 2002). FasL also plays a role in the UtA remodeling (Bai et al., 2009). These mechanisms may be related to the EO-PE and the increased EV expression of FasL in EO-PE.

Regarding TRAIL, this study shows an increased TRAIL/EV proportion in the EO-PE group. This increase may be a response to STB reduction or functional loss caused by the altered apoptosis process during PE (Bai et al., 2009). It also may derive from the increased apoptosis induction in activated lymphocytes as a defense mechanism against fetal allograft rejection by the maternal immune system (Stenqvist et al., 2013).

The apoptosis-inducing capability of the isolated EV was assessed using the TUNEL assay. Placental EV has been reported to induce apoptosis in T cells as an immunological tolerance

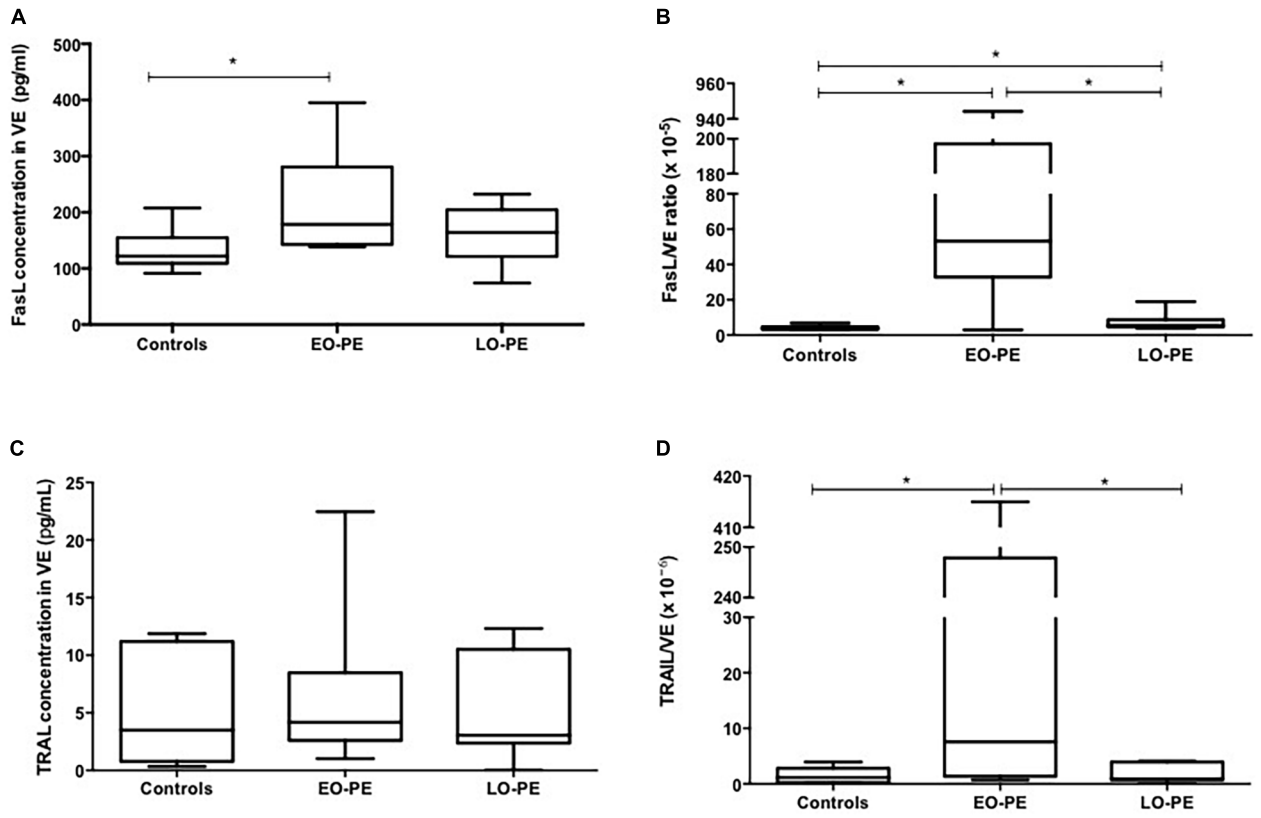

FIGURE 2 | FasL and TRAIL quantification in VE. **(A)** Quantification of FasL in VE (controls vs. EO-PE p = 0,0379; controls vs. LO-PE p = 0,2564; EO-PE vs. LO-PE: p = 0,6031). **(B)** FasL/VE ratio (controls vs. EO-PE p = 0,0262; controls vs. LO-PE p = 0,0379; EO-PE vs. LO-PE: p = 0,0262). **(C)** Quantification of TRAIL in VE (controls vs. EO-PE p = 0,6894; controls vs. LO-PE p = 0,7791; EO-PE vs. LO-PE: p = 0,6894). **(D)** TRAIL/VE ratio (controls vs. EO-PE p = 0,0379; controls vs. LO-PE p = 0,9656; EO-PE vs. LO-PE: p = 0,0379). *$p \leq 0,05$.

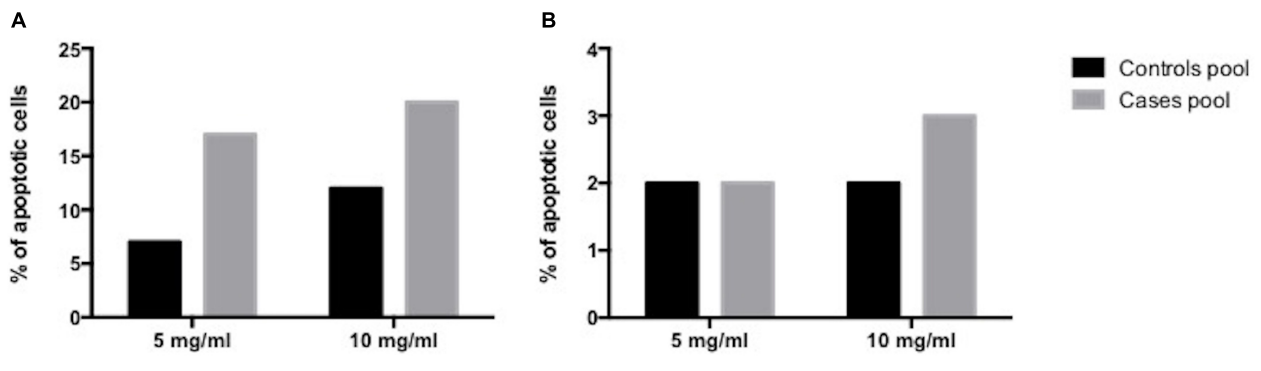

FIGURE 3 | Human placenta-derived VE apoptosis induction analysis. **(A)** at 8 h **(B)** at 24 h.

mechanism. For that reason, this experimental model (Sabapatha et al., 2006) used Jurkat T cells. Leukemic cells have been reported to express FasL on their surfaces, so they were also used in this model (Yamada et al., 2017). Previous *in vitro* studies have shown that EV from placenta and serum of healthy pregnant women induce a significant increase (3.38 fold) of Jurkat T cells apoptosis compared to EV isolated from the serum of non-pregnant women. Studies also show that apoptosis induction depends on the Fas/FasL complex (Gercel-Taylor et al., 2018). Gupta *et al.* in turn, showed that EV derived from explants cultures of placentas from uncomplicated pregnancies may play a role in the response of activated T cells (EV reduce T-lymphocyte proliferation and production of IL-2 and IFNγ). Those authors, however, did not find apoptosis induction in the cells (Gupta et al., 2005). Results of the present study show that the isolated EV are capable of triggering apoptosis in

Jurkat T cells in a concentration-dependent manner (Stenqvist et al., 2013). There is also a higher apoptosis induction by EV from placentas of women with PE. This may result from EV triggering higher protection for the fetoplacental unit from activated maternal immunecells (Nair and Salomon, 2018). The higher apoptosis induction may also result from the inhibition of T lymphocyte activation and prolipheration (Gupta et al., 2005). A higher EV apoptosis-inducing capability may suggest EV content regulation aiming for higher apoptosis in target cells (Gilani et al., 2016). On the other hand, analysis of apoptosis with different treatments at 8 h and 24 h shows a reduction in apoptosis percentage at 24 h. *Zhang et al.,* working from studies reporting T lymphocyte phagocytic capacity, exposed Mycobacterium tuberculosis (H37Ra) to Jurkat T cells, noting some morphological changes in the Jurkat T cells (cytoskeleton remodeling, wrinkled cellular surfaces, pseudopodia formation) associated with the induction of a form of non-selective endocytosis named macropinocytosis (Zhang et al., 2015).The eventual phagocytic capacity of the Jurkat T cells may explain the reduction in the apoptotic cells percentage over time.

This is the first study to evaluate EV FasL and TRAIL levels from placentas with EO-PE and LO-PE and to assess their apoptosis-inducing capability. The principal limitation of this study is the sample size. It is recommended to replicate methods used in this study with a larger sample. Unfortunately, it is not possible to establish with certainty the nature of the isolated EV. Their size corresponds to exosomes, and analysis revealed the presence of CD63, a principal exosome marker (Zhang et al., 2019). This marker, however, has also been found in other EV, such as micro-particles (Rank et al., 2012) and apoptotic bodies (Crescitelli et al., 2013). The results in this study would have been complemented by measurement of FasL and TRAIL expression in placentas. A limited number of samples was a weakness that kept the apoptosis essays from reaching conclusions at a statistically significant level and from comparing EO-PE and LO-PE. Unfortunately, no replicas of the apotosis induction experiments were possible, due to the availability of extracellular vesicle samples, for this reason it is recommended to carry out new studies to replicate these results.

This study suggests that FasL and TRAIL molecules, present in EV and of placental origin, participate in the pathophysiology of EO-PE and LO-PE. Study results provide additional evidence of a possible role of EV in the immune tolerance regulation in normal pregnancy and in pathological conditions such as PE, probably also with participation of FasL and TRAIL. It is necessary to continue research on this subject to gain more knowledge. It will be important to precisely identify the immunological target cells and the molecular mechanisms involved. There is also a conundrum: Are other cell types, such as the maternal vascular endothelial cells, a target? If so, what is their possible role in PE?

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hospital Universitario San Ignacio-Faculty of Medicine of Pontificia Universidad Javeriana of Bogotá, Colombia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PA-R and RG-R conceived, designed, planned, and supervised the experiments. SQ and AB supported the implementation of the apotosis and extracellular vesicle isolation experiment. CM-A and TG performed the experiments. MO-C and JS collected samples and data of patients. CM-A, PA-R, and RG-R processed and analyzed the data and drafted the manuscript. All authors provided critical feedback, contributed to the interpretation of the results, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.708824/full#supplementary-material

Supplementary Figure 1 | Human placenta-derived VE apoptosis induction plots at 8 h. **(A)** Selection of the population, **(B)** Elimination of duplets, **(C)** negative control, **(D)** positive control (DMSO), **(E)** VE control 5mg/mL, **(F)** VE control 10mg/mL, **(G)** VE pool of preeclampsia cases 5 mg/mL, **(H)** VE pool of preeclampsia cases 10 mg/mL.

Supplementary Figure 2 | Human placenta-derived VE apoptosis induction plots at 24 h. **(A)** Selection of the population, **(B)** Elimination of duplets, **(C)** negative control, **(D)** positive control (DMSO), **(E)** VE control 5 mg/mL, **(F)** VE control 10 mg/mL, **(G)** VE pool of preeclampsia cases 5 mg/mL, **(H)** VE pool of preeclampsia cases 10 mg/mL.

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ORIGINAL RESEARCH
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Congenital Anomalies Programmed by Maternal Diabetes and Obesity on Offspring of Rats

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Embryo-fetal exposure to maternal disorders during intrauterine life programs long-term consequences for the health and illness of offspring. In this study, we evaluated whether mild diabetic rats that were given high-fat/high-sugar (HF/HS) diet presented maternal and fetal changes at term pregnancy. Female rats received citrate buffer (non-diabetic-ND) or streptozotocin (diabetic-D) after birth. According to the oral glucose tolerance test (OGTT), the experimental groups (n = 11 animals/group) were composed of non-diabetic and diabetic receiving standard diet (S) or HF/HS diet. High-fat/high-sugar diet (30% kcal of lard) in chow and water containing 5% sucrose and given 1 month before mating and during pregnancy. During and at the end of pregnancy, obesity and diabetes features were determined. After laparotomy, blood samples, periovarian fat, and uterine content were collected. The diabetic rats presented a higher glycemia and percentage of embryonic losses when compared with the NDS group. Rats DHF/HS presented increased obesogenic index, caloric intake, and periovarian fat weight and reduced gravid uterus weight in relation to the other groups. Besides, this association might lead to the inflammatory process, confirmed by leukocytosis. Obese rats (NDHF/HS and DHF/HS) showed higher triglyceride levels and their offspring with lower fetal weight and ossification sites, indicating intrauterine growth restriction. This finding may contribute to vascular alterations related to long-term hypertensive disorders in adult offspring. The fetuses from diabetic dams showed higher percentages of skeletal abnormalities, and DHF/HS dams still had a higher rate of anomalous fetuses. Thus, maternal diabetes and/or obesity induces maternal metabolic disorders that contribute to affect fetal development and growth.

Keywords: hyperglycemia, obesity, pregnancy, biochemical, malformation, rat

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INTRODUCTION

Diabetes mellitus (DM) is a syndrome that is a growing health problem, accounting for 10.4% of global mortality. In 2015, hyperglycemia during pregnancy was observed in 16.2% of women (Cho et al., 2018). In the first few weeks of pregnancy, maternal diabetes is intensely linked to higher number of spontaneous abortions and major congenital malformations (Kitzmiller et al., 1996; Ray et al., 2001).

Fetal programming is a theory that suggests that the environment around the developing fetus plays an important role in determining the risk of disease in childhood and adulthood (Entringer et al., 2012). In this sense, factors such as overweight, obesity, and maternal diabetes during pregnancy are known to be effective agents leading to chronic-disease development in offspring (Yessoufou and Moutairou, 2011), showing the relevance of intrauterine environment for health of descendants in the future.

To reproduce maternal hyperglycemia found in Type 2 DM in animal models, streptozotocin (STZ) induction can be performed in the neonatal period of rats (Tsuji et al., 1988; Jawerbaum and White, 2010; Santos et al., 2015; Bequer et al., 2018; Bueno et al., 2020). This type of experimental diabetes is termed as "mild diabetes" (Hauschildt et al., 2018; Machado et al., 2020). Besides diabetic status, a change in lifestyle, especially in dietary patterns related with growing consumption of industrialized foods (high in calories and fat), affects diabetic progress at long term (Hu, 2011; Ley et al., 2014; Popkin, 2015; Krishan et al., 2018). Animals that are fed high-fat (HF) and/or high-sugar (HS) diets showed metabolic changes, such as increased concentrations of glucose, triglycerides (TG), total cholesterol (TC), and obesity (Matias et al., 2018; Zhao et al., 2019). The offspring of mothers who consumed the HF/HS diet had greater fat tissue, glycemia, TG, and TC levels (Martins Terra et al., 2020). Despite the knowledge about the single effect of HF/HS diet and diabetes on the metabolic response in animals, there are still few studies exploring the association of these two variables during pregnancy. Considering that pregnancy is a critical period, where maternal conditions and habits can lead to persistent changes in offspring (Fleming et al., 2015), it is important that the studies are conducted in a manner where new care strategies can be taken.

Thus, the hypothesis of this study is that diabetic rats submitted to the HF/HS diet before and during pregnancy will present exacerbated damage on a biochemical profile, leading to impaired maternal-fetal relationship. Therefore, the aim of this study was to evaluate maternal and fetal repercussions of the diabetes associated with an HF/HS diet offered before and during pregnancy of rats.

MATERIALS AND METHODS

Animals

Female Wistar rats (230 ± 250 g) were obtained from the Center for Maintenance of Experimental Animals of our Institution, and were maintained under standard laboratory conditions (22 ± 3°C, 12-h light/dark cycle), with pelleted food (Purina rat chow, Purina®, São Paulo State, Brazil) and tap water *ad libitum*. The local Ethical Committee for Animal Research authorized and approved all the procedures and animal handling (Protocol No. 23108.022251/2019-61).

After 1 week of acclimatization, the females were mated with the male rats with similar age (ratio 3:1) to obtain offspring for induction of diabetes. The experimental sequence of the experiment is summarized in **Figure 1**.

Induction and Confirmation of Diabetes

For diabetes (D) induction, half of each female litter was injected with Streptozotocin (100 mg/kg, sc., Sigma–Aldrich, St. Louis, MO, USA) diluted in citrate buffer (.1 mol/L, pH 4.5) on the first day of life (24 h after delivery) (Soares et al., 2021) to induce beta cell necrosis, reproducing glycemic levels similar to Type 2 DM. Non-diabetic (ND) animals were injected with only citrate buffer to simulate the conditions of the STZ-induced group. At day 90 of life, oral glucose tolerance test (OGTT) was performed for inclusion or exclusion of rats to ND and D groups. This test is a marker routinely used in the clinic for diagnosing diabetic status. Then, the rats were fasted for 6 h; after which, a drop of blood was collected from the tail of the rats to determine glycemia (time 0), using a conventional glucometer. The rats were intragastrically given glucose solution (.2 g/m) at a dose of 2-g/kg body weight and after 30, 60, and 120 min later, the blood glucose levels were determined (Neto et al., 2020).

In order to determine the inclusion and exclusion criteria, the standards established by Gallego et al. (2018) were used, and modified from diabetes classification parameters suggested by the American Diabetes Association (2020). For the control group, only the rats with glycemia $<$ 140 mg/dL in different time points during OGTT were included. For the diabetic group, the rats presenting least one-time point with glycemia $\geq$ 200 mg/dL after overload glucose during OGTT were included in the group. The female animals that did not present OGTT with these characteristics for inclusion in the control or diabetic group were excluded and euthanized.

Experimental Groups

Considering the four experimental groups, and based on previous experiments conducted in our laboratory in relation to reproductive parameters, using 90% power and error type I of 5%, the effect size was determined. Based on the effect size, the sample size was 11 rats per group.

After inclusion and exclusion criteria, the rats (90 days of life = adulthood) were randomized in the experimental groups: non-diabetic rats that received standard diet (NDS); non-diabetic rats receiving high-fat/high-sugar diet (NDHF/HS); diabetic rats, given standard diet (DS); and diabetic rats that received high-fat/high-sugar (HF/HS) diet.

Standard or High-Fat/High-Sugar Diet

Females from non-diabetic and diabetic dams randomly received standard diet (commercial food: 28.54% Kcal of protein, 62.65% Kcal of carbohydrate, 8.7% Kcal of fat (Purina rat chow, Purina®, Brazil) or high-fat diet (23.43% Kcal of protein, 46.63% Kcal of carbohydrate, 30% Kcal of fat) according to the experimental group (**Table 1**). The main source of fat consisted of lard. After preparation, the feed was kept refrigerated until the time of consumption by the animals. In addition, the rats given high-fat diet groups also received water with 5% sucrose (high sugar) during the same period from day 90 to 120 of life and during pregnancy, which corresponds to reproductive age of adult rats.

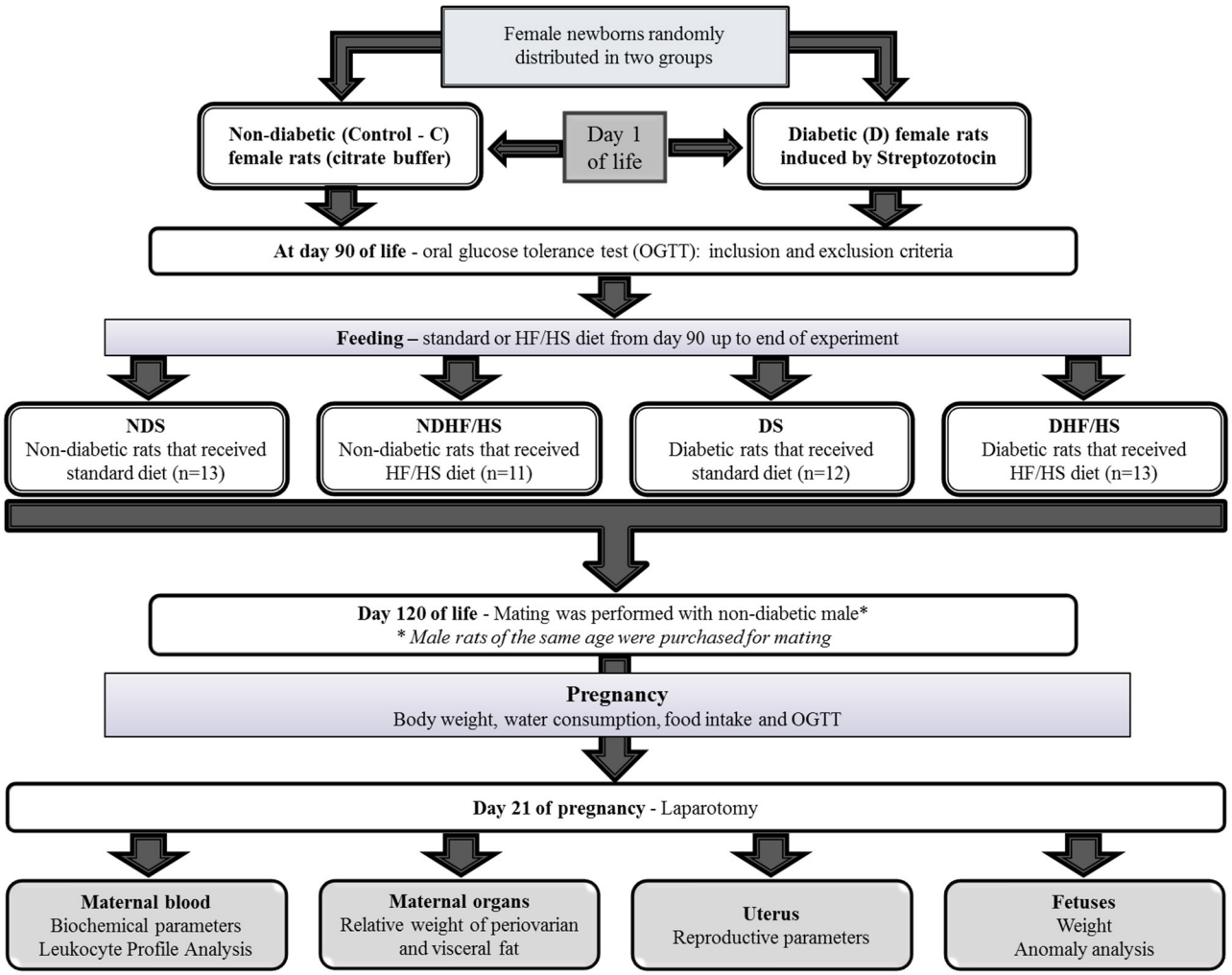

FIGURE 1 | Experimental design.

TABLE 1 | Nutritional values of diet offered to non-diabetic and diabetic rats.

Information	Food (Kcal/g)		Water (Kcal/mL)	
	Commercial	High-fat	Standard	High-sugar
Gross energy	4.02	4.93	–	0.20
Mixture (%)	7.45	3.65	100	100
Dry matter (%)	92.55	96.35	–	–
Mineral matter (%)	6.66	2.84	–	–
Crude protein (%)	25.76	26.77	–	–
Ether extract (%)	3.49	15.19	–	–
Gross fiber (%)	43.63	45.42	–	–
Carbohydrates (%)	13.01	6.13	–	5.00

Mating

At 120 days of life, the female rats were similarly mated as their mothers. After 15 consecutive days, non-mated rats were considered infertile and excluded from the experiment. For this, three females were placed in the overnight period with normoglycemic males presenting similar age, which were purchased for this purpose. The next morning (7–9 a.m.) the male arts were removed and vaginal smears were performed in

female rats. The presence of spermatozoa on the slides confirmed the diagnosis of pregnancy, which was considered zero pregnancy day (D0) (Damasceno et al., 2011).

Course of Pregnancy—Diabetes and Obesity Features

Maternal body weight, food consumption, and water intake were measured every 7 days up to the end of pregnancy, at approximately 9 a.m. At days 0 and 17 of pregnancy, OGTT was again performed to evaluate glycemia. The glycemic values were used to mathematically estimate the total area under the curve (AUC) by the trapezoidal method (Tai, 1994; Gallego et al., 2019). For the obesity parameter, Lee Index was obtained at days 0 and 17 of pregnancy, and defined as the cube root of body weight (g) 10/nasoanal length (cm), for which a value equal to or <0.300 was classified as normal. Rats presenting values higher than 0.300 were classified as obese (Bernardis and Patterson, 1968; Soares et al., 2017).

At term pregnancy (day 21), the female rats were anesthetized with sodium thiopental (Thiopentax®, intraperitoneal route, 120 mg/kg according to protocols of Ethical Committee), and, after confirming the signs that showed successful anesthetic procedure, the animals were decapitated to obtain blood samples. Then, the rats were submitted to laparotomy for exposure of uterine horns. White adipose depots were collected around ovaries and then weighed.

Biochemical and Hematological Profile Analysis

The blood samples were collected in dry tubes and maintained on ice for 30 min and then centrifuged at 1,575 × *g* for 10 min at 4°C. The serum supernatant was at −80°C for determination of triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c), using commercial kits.

For hematological analysis, blood was collected (500 μL) and transferred to tubes with anticoagulant (EDTA). The total leukocyte count was determined on blood samples diluted 1:20 in Turk's solution, using a Neubauer's hemocytometer. For differential white blood cell counting, blood smears were fixed with methanol and stained with Giemsa's solution. According to staining and morphological criteria, differential cell analysis was performed under the light microscope by counting 100 cells, and the percentage of each cell type was calculated.

Reproductive Outcomes and Fetal Development

The gravid uterus was withdrawn and dissected for evaluation of live and dead fetuses, reabsorption (embryonic death), implantation, and corpora lutea numbers. The number of undetectable implantation sites was determined by the Salewski method (Salewski, 1964). The percentage of preimplantation loss was calculated by [(number of corpora lutea – number of implantation)/number of corpora lutea] × 100. The percentage of postimplantation loss was determined by [(number of implantation – number of live fetuses)/number of implantation] × 100 (Afiune et al., 2017). Following the collection of fetuses from the uterine horns, these were weighed and classified as small (SGA), adequate (AGA), or large (LGA) for gestational age (Moraes-Souza et al., 2017). The placentas were weighed to calculate the placental efficiency (fetal weight/placental weight) (Volpato et al., 2015).

After weight, each fetus was externally examined for cranial conformation, implantation of ears, eyes, and mouth (existence of a cleft lip), anterior and posterior limbs (absence or excess of fingers, position, and size of limbs), thoracic, abdominal, and dorsal regions (presence of hemorrhage, hematoma, and neural tube closure defect), tail (size and shape), and anal perforation. Half of the number of fetuses of each liter was fixed in Bodian's solution, and serial sections were prepared as described by Wilson (1965) for visceral examination. The other fetuses were processed for examination of the bones by the staining procedure of Staples and Schnell (1964). Besides the skeletal analyses, the counting of the ossification sites was performed according to methodology proposed by Aliverti et al. (1979), which determines the degree of fetal development. Fetuses that showed no external, skeletal, and visceral anomalies were considered normal.

Statistical Analysis

The comparison of the mean values between the experimental groups was determined by analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Student's *t*-test was used to compare difference of time (day 0 × day 21 of pregnancy). Proportions were calculated by the Fisher's exact test. To verify the normality of the results, the Shapiro–Wilk Normality test was used. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Obesity Features

Table 2 shows obesity features. The rats NDHF/HS presented lower feed intake and higher water intake, a positive obesity rate at day 0 of pregnancy, periovarian, and visceral adipose tissue weight compared with the NDS group. The DS group showed decreased gravid uterus weight and higher periovarian/visceral adipose tissues weight when compared with NDS rats. The DHF/HS rats presented increase in water and caloric intake, number of obese rats, periovarian and visceral adipose tissue weight, decrease in feed intake, maternal weight gain, and gravid uterus weight compared with the NDS rats. In addition, the feed intake was increased, and water intake was decreased compared with NDHF/HS and DS groups; and the DHF/HS group had the gravid uterus weight decrease compared with the NDHF/HS rats and a higher positive obesity rate compared with the DS group.

Diabetes Biomarker

The area under the curve (AUC) obtained by oral glucose tolerance test (OGTT) was increased in both diabetic groups (DS and DHF/HS) on days 0 and 17 of pregnancy compared with non-diabetic groups (NDS and NDHF/HS). In addition, the DHF/HS group showed an increase in AUC on day 17 of pregnancy compared with the DS group. There was no difference

TABLE 2 | Obesity features of non-diabetic (ND) and diabetic (D) rats treated or not (S) with high-fat/high-sugar diet (HF/HS) before and during pregnancy.

	Groups			
	NDS ($n = 13$)	**NDHF/HS ($n = 11$)**	**DS ($n = 12$)**	**DHF/HS ($n = 13$)**
Food intake (g/day)[a]	19.7 ± 1.6	13.5 ± 1.5*	21.0 ± 2.4	16.4 ± 3.1*#$
Water intake (mL/day)[a]	44.2 ± 5.1	92.1 ± 23.3*	39.4 ± 13.9	72.0 ±18.8*#$
Caloric intake (Kcal/day)[a]	79.2 ± 6.4	84.9 ± 7.8	84.4 ± 9.6	95.2 ± 15.8*
Weight gain in pregnancy (g)[a]	116.7 ± 13.3	110.8 ± 16.6	90.7 ± 42.3	79.8 ± 39.9*
Gravid uterus weight (g)[a]	82.3 ± 13.2	82.9 ± 13.0	55.4 ± 31.4*	55.8 ± 26.9*#
Positive obesity (%)[b]				
Previous pregnancy	0.0	71.4*	25.0#	77.8*$
Weight of periovarian adipose tissue (g)[a]	0.4 ± 0.1	0.9± 0.2*	0.8 ± 0.2*	1.1 ± 0.4*
Weight of visceral adipose tissue (g)[a]	3.1 ± 0.5	4.0 ± 1.5*	3.9 ± 0.9*	5.4 ± 1.8*

*Data shown as mean ± standard deviation (SD) and proportions (%). *$p < 0.05$, compared with the NDS group; #$p < 0.05$, compared with the NDHF/HS group; $$p < 0.05$, compared with the DS group ([a]ANOVA followed Tukey's multiple comparison test; [b]Fisher's exact test).*

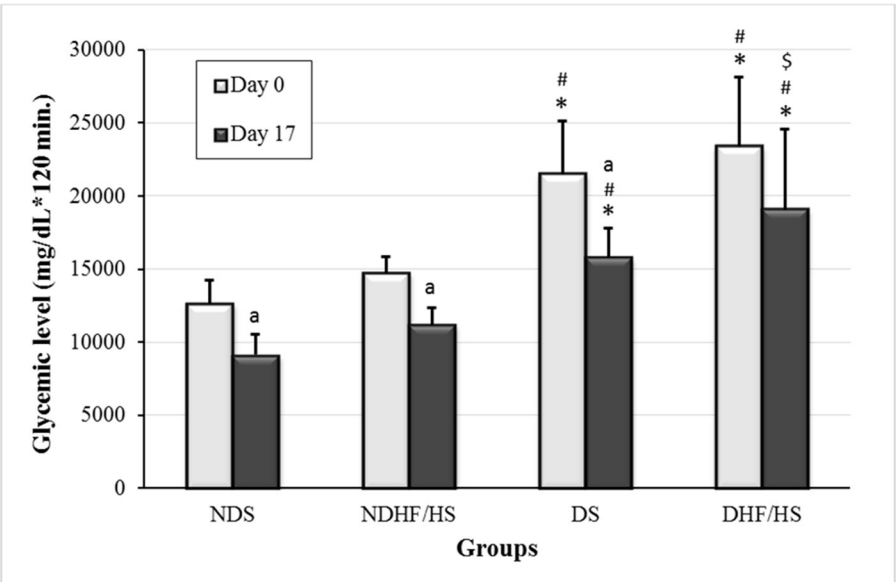

FIGURE 2 | An area under the curve of oral glucose tolerance test (OGTT) on days 0 and 17 of pregnancy of non-diabetic (ND) and diabetic (D) rats treated or not (S), with high-fat/high-sugar diet (HF/HS) before and during pregnancy. Data shown as mean ± standard deviation (SD). *$p < 0.05$, compared with the NDS group; #$p < 0.05$, compared with NDHF/HS group; $$p < 0.05$, compared with the DS group (ANOVA followed Tukey's Multiple Comparison test); [a]$p < 0.05$, compared with day 0 of pregnancy (Student's *T*-test).

in AUC between days 0 and 17 of pregnancy of DHF/HS rats (**Figure 2**).

Maternal Biochemical Parameters

There was no difference in TC, ALT, and AST concentrations among the groups. The DHF/HS group showed an increase in protein and albumin compared with the non-diabetic groups (NDS and NDHF/HS). The TG levels groups presented an increase in the NDHF/HS group compared with standard diet groups (NDS and DS), and the DHF/HS showed an increase in relation to other groups (**Table 3**).

Hematological Profile

The DS group showed decreased number of monocytes compared with the NDS rats. There was an increase in total leukocytes, segmented, and eosinophil in the DHF/HS group compared with the other experimental groups. The DHF/HS group also had increased number of monocytes in relation to the NDHF/HS and DS groups (**Table 4**).

Pre- and Postimplantation Embryonic Losses

Figure 3 shows the embryonic losses before and after the implantation process. The diabetic rats of both groups (DS and DHF/HS) showed an increased percentage of pre- and

TABLE 3 | Biochemistry parameters at term pregnancy of non-diabetic (ND) and diabetic (D) rats treated or not (S) with high-fat/high-sugar diet (HF/HS) before and during pregnancy.

	Groups			
	NDS ($n = 13$)	**NDHF/HS ($n = 11$)**	**DS ($n = 12$)**	**DHF/HS ($n = 13$)**
Total protein (g/dL)	5.5 ± 0.9	5.5 ± 1.5	6.3 ± 1.1	8.1 ± 1.9$^{*\#}$
Albumin (mg dL)	2.8 ± 0.2	2.8 ± 0.4	3.1 ± 0.5	3.3 ± 0.4$^{*\#}$
TC (mg/dL)	61.4 ± 3.2	77.4 ± 12.4	66.0 ± 5.2	82.0 ± 9.8
TG (mg/dL)	97.2 ± 7.1	372.4 ± 119.9*	182.0 ± 34.3$^{\#}$	485.6 ± 138.5$^{*\#\$}$
ALT (U/l)	87.2 ± 8.9	90.8 ± 6.3	84.0 ± 7.8	92.6 ± 10.8
AST (U/L)	188.4 ± 8.5	151.8 ± 21.6	181.8 ± 27.6	164.6 ± 30.9

*Data shown as mean ± standard deviation (SD). *$p < 0.05$, compared with the NDS group; $^{\#}p < 0.05$, compared with the NDHF/HS group; $^{\$}p < 0.05$, compared with the DS group (ANOVA followed Tukey's multiple comparison test).*

TABLE 4 | A hematological profile at term pregnancy of non-diabetic (ND) and diabetic (D) rats treated or not (S) with high-fat/high-sugar diet (HF/HS) before and during pregnancy.

	Groups			
	NDS ($n = 13$)	**NDHF/HS ($n = 11$)**	**DS ($n = 12$)**	**DHF/HS ($n = 13$)**
Leukocytes (10^3/mm^3)	5.91 ± 0.82	5.47 ± 2.63	6.03 ± 0.82	8.64 ±1.65$^{*\#\$}$
Segmented (10^3/mm^3)	2.23 ± 0.31 (38–43%)	2.64 ± 1.60 (41–51%)	2.52 ± 0.43 (33–53%)	4.51 ± 1.24$^{*\#\$}$ (44–60%)
Lymphocytes (10^3/mm^3)	3.41 ± 0.73 (52–62%)	2.73 ± 1.01 (45–55%)	3.34 ± 1.02 (44–64%)	3.81 ± 1.01 (35–53%)
Monocytes (10^3/mm^3)	0.20 ± 0.08 (2–4%)	0.12 ± 0.06 (1–3%)	0.09 ± 0.04* (0–2%)	0.25 ± 0.08$^{\#\$}$ (2–4%)
Eosinophils (10^3/mm^3)	0.01 ± 0.02 (0–1%)	0.01 ± 0.01 (0–1%)	0.02 ± 0.03 (0–1%)	0.09 ± 0.08$^{*\#\$}$ (0–2%)
Basophil (10^3/mm^3)	0.00 ± 0.00 (0–0%)	0.00 ± 0.00 (0–0%)	0.00 ± 0.00 (0–0%)	0.00 ± 0.00 (0–0%)

*Data shown as mean ± standard deviation (SD). *$p < 0.05$, compared with the ND group; $^{\#}p < 0.05$, compared with the NDHF/HS group; $^{\$}p < 0.05$, compared with the DS group (ANOVA followed Tukey's multiple comparison test).*

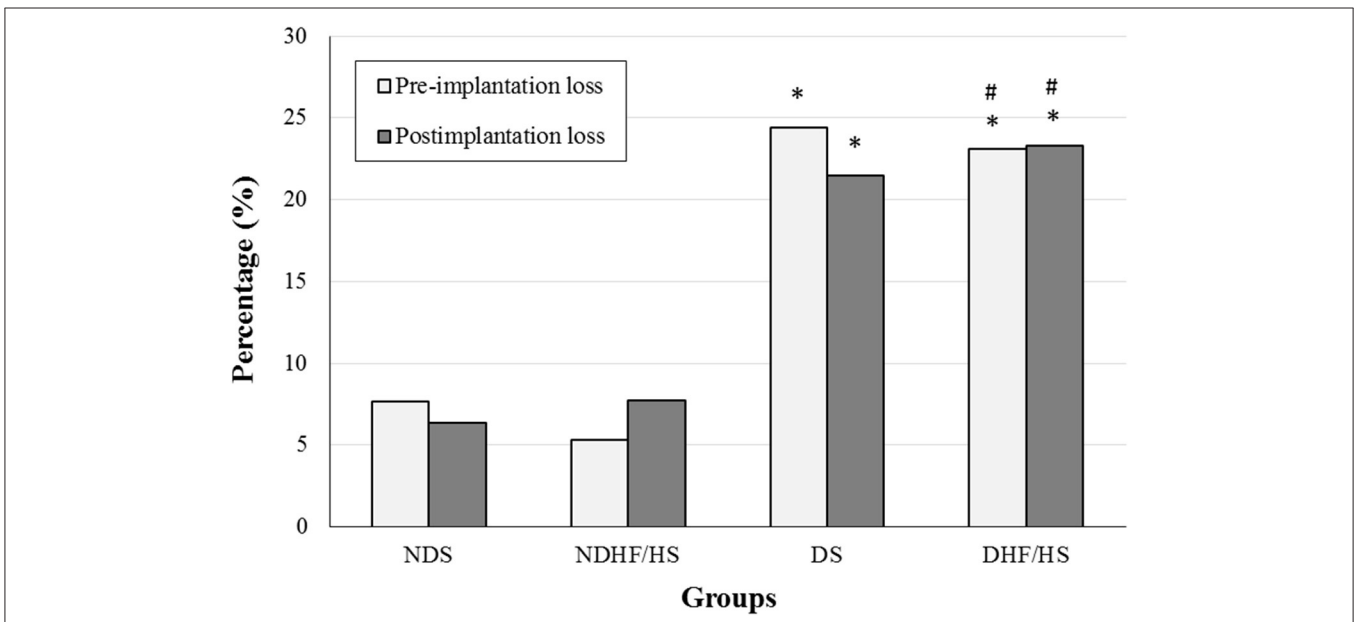

FIGURE 3 | Percentage (%) of pre- and postimplantation losses of non-diabetic (ND) and diabetic (D) rats treated or not (S), with high-fat/high-sugar diet (HF/HS) before and during pregnancy. *$p < 0.05$, compared with the NDS group; $^{\#}p < 0.05$, compared with the NDHF/HS group (Fisher's exact test).

postimplantation losses compared with those of non-diabetic animals (NDS and NDHF/HS).

Fetal and Placental Data

The fetal weight and number of fetuses classified as adequate for gestational age (AGA) were decreased in the NDHF/HS and DHF/HS groups compared with those of NDS group. The percentage of fetuses classified as small for gestational age (SGA) was increased in the NDHF/HS and DHF/HS rats in relation with those of the NDS group. The NDHF/HS group presented decrease in placental weight compared with the other groups, and the DHF/HS rats showed decrease in placental efficiency compared with the NDS rats. The ossification sites of fetuses from dams that received HF/HS diet (ND and D) were decreased in relation to respective control groups (**Table 5**).

Fetal Anomalies

The percentage of normal fetuses was decreased in three groups when compared with the ND group. The diabetic groups (DS and DHF/HS) showed an increased percentage of fetuses with skeletal anomalies compared with the ND groups. The fetuses of the DHF/HS rats showed higher incidence of visceral anomalies compared with those of the ND group, higher percentage of skeletal anomalies and lower percentage of normal fetuses compared with those of the NDHF/HS group (**Figure 4A**). Representative images of the main anomalies found are shown in **Figures 4B–J**.

DISCUSSION

The streptozotocin-induced mild diabetes in rat offspring after birth caused a diabetic status. This was confirmed by oral glucose tolerance test (OGTT) and higher area under curve (AUC) data. Before pregnancy, there was no significant difference in fertility rates among the groups. In the experimental model of mild diabetes induction, used in the present study, the fertility rate was around 90%, corroborating Sinzato et al. (2021). In the groups that consumed HS/HF diet, the treatment time was not enough to change the fertility rate. During pregnancy of these rats, the hyperglycemia led to impairment on embryonic development, contributing to embryo losses as verified at term pregnancy. The rats that received high-fat diet and sugar in drinking water at adulthood presented with greater Lee index values, confirming obesity. In addition, these female rats showed higher periovarian and visceral fat weight and hypertriglyceridemia. In this maternal condition, there was higher incidence of small fetuses for gestational age, indicating intrauterine growth restriction (IUGR) associated with obesity. The use of animal models to study maternal association between diabetes and obesity helps understand the functional, biochemical, and morphological changes caused by these connected diseases. Our findings showed that diabetes and obesity status caused maternal hyperglycemia and an abnormal leukocyte profile, contributing to IUGR. In addition, this association led to embryo-fetal losses and the onset of anomalies in the fetuses at the end of pregnancy, confirming the maternal, fetal, and perinatal complications induced by diabetes and obesity during pregnancy.

Metabolic disorders, such as diabetes, may cause hyperphagia condition, but the mechanisms involved are not fully understood (Li et al., 2019). The regulator of food intake is influenced by the balance among appetite, satiety, and energy expenditure, and this biological process is called "energy homeostasis" (Morton et al., 2014; Deemer et al., 2019). This balance is regulated by the central nervous system (Deemer et al., 2019) and multiple metabolic signals, such as leptin (Zhang and Chua, 2017), insulin (Brüning et al., 2000), glucagon-like peptide 1 (GLP1) (Ong et al., 2017), and cholecystokinin (CCK) (Woods et al., 2018). In this study, diabetes and high-fat/high-sugar diet alone did not interfere with daily caloric intake. However, the association between diabetes and abnormal diet might activate neurocircuits, which impaired the controller system of energy homeostasis, influencing in body weight (Morton et al., 2014) and the regulation of caloric intake. Then, even the rats eating less high-fat diet but drinking more sugar in the water presented obesity status. The deregulation of energy consumption is one of the major causes of obesity (Erlanson-Albertsson, 2005), confirmed by body weight (de Almeida et al., 2016), body composition and fat deposits, especially visceral fat (Poirier et al., 2006). Our findings showed that diabetic rats submitted to a high-fat/high-sugar diet showed a reduction in body weight gain during pregnancy, but, even so, they developed obesity, as verified by Lee index, which is a murinometric parameter for obesity classification used in experimental studies (Bernardis and Patterson, 1968; Fernandes et al., 2012). Concomitantly, there was an increased weight of periovarian and visceral fat. In experimental models, the carcass relative fat is one of the variables to indicate obesity (Nascimento et al., 2008; Kim et al., 2017).

The oral glucose tolerance test (OGTT) determines degree of glucose tolerance, expressing the ability of β-pancreatic cells to secrete insulin and tissue sensitivity to this hormone (American Diabetes Association, 2020). There were higher blood glucose values in the OGTT in diabetic groups and consequent increase in the area under the curve (AUC), leading to glucose intolerance and, later, diabetes. Then, once diabetes and obesity have been confirmed, it was demonstrated that diabetic and obese rats presented higher levels of blood total protein and albumin. These biochemical parameters are used in animal nutrition research to evaluate its health (Luca and Reis, 2004). Several processes regulate plasma albumin concentration, including synthesis, distribution, and exogenous albumin loss (Dom and Kaysen, 2003). Roche et al. (2008) and Guerin-Dubourg et al. (2012) described albumin as an antioxidant, and it might be elevated in our animals to compensate the higher levels of reactive oxygen species (ROS) induced by diabetes (Raza et al., 2011; Patche et al., 2017; Sinzato et al., 2019) and obesity (Diniz et al., 2004; Burneiko et al., 2006; De Sibio et al., 2013). Our results showed that obesity, alone or associated with diabetes, causes dyslipidemia. Other authors also verified dyslipidemia in experimental animals (Panchal et al., 2011; Zhou et al., 2014; Hao et al., 2015; Senaphan et al., 2015), and different types of diets influence the lipid profile (Desroches et al., 2006). Lipid metabolism, including lipid absorption, transport, synthesis, and degradation, is a complex process, which can lead to other diseases (Huang and Freter, 2015). Among these, diabetes (Dong et al., 2017), inflammation,

TABLE 5 | Fetal and placental weights, placental efficiency, and ossification sites of fetuses from non-diabetic (ND) and diabetic (D) rats treated or not (S) with high-fat/high-sugar diet (HF/HS) before and during pregnancy.

	Groups			
	NDS (n = 147 fetuses)	**NDHF/HS (n = 131 fetuses)**	**DS (n = 95 fetuses)**	**DHF/HS (n = 102 fetuses)**
Fetal weight (g)[a]	5.57 ± 0.46	5.25 ± 0.65*	5.46 ± 0.49	5.35 ± 0.55*
SGA Fetuses (%)[b]	4.08	19.08*	10.54	16.66*
AGA Fetuses (%)[b]	90.48	75.58*	84.20	80.40*
LGA Fetuses (%)[b]	5.44	5.34	3.16	2.94
Placental weight (g)[a]	0.49 ± 0.06	0.47 ± 0.07*	0.49 ± 0.08#	0.50 ± 0.09#
Placental efficiency[a]	11.58 ± 1.42	11.45 ± 1.85	11.24 ± 1.68	10.82 ± 1.73*
Ossification sites[a]	24.96 ± 1.71	22.80 ± 2.02*	24.42 ± 2.49	21.78 ± 0.93*$

*Data shown as mean ± standard deviation (SD) and proportions (%). *$p < 0.05$, compared with the C Group; #$p < 0.05$, compared with the NDHF/HS group; $$p < 0.05$, compared with the D Group ([a]ANOVA followed Tukey's multiple comparison test; [b]Fisher's exact test).*

atherosclerosis (Joseph et al., 2003), obesity (Kaess et al., 2014), and hypertension (Siri-Tarino and Krauss, 2016) are related. The male rats feeding HFD showed increased blood pressure (Sá et al., 2019), and Hsu et al. (2019) showed that the consumption of HFD during pregnancy of rats was responsible for inducing hypertension in adult offspring.

Considering the hematological profile, the association between diabetes and obesity increased the number of total leukocytes, segmented (mature neutrophils), monocytes, and eosinophil. The total and differential leukocyte count is an important parameter to evaluate conditions related with inflammatory processes (George-Gay and Parker, 2003). Increased leukocyte amount due to deregulation of immune activity caused by adipose tissue expansion contributes to obesity-induced inflammation (Trellakis et al., 2012; Poret et al., 2018). Obesity may cause immunomodulation, inducing a higher ratio from neutrophils to lymphocytes due to increased recruitment and activation of peripheral blood neutrophils to adipose tissue (Elgazar-Carmon et al., 2008; Trellakis et al., 2012). In addition, it can stimulate mobilization of bone marrow monocytes so that they fall into the bloodstream and reach adipose tissue as macrophages (Ghigliotti et al., 2014). In this study, the diabetic rats presented lower levels of monocytes. Monocyte is one of the main leukocyte subtypes and is considered an inflammatory biomarker (Badr et al., 2019), and its influx in perivascular regions and retinal pigment epithelium has been verified (Benhar et al., 2016). Decreased peripheral blood monocyte levels were related to diabetic retinopathy in diabetic adults without potential confounders (Wan et al., 2020), suggesting the onset of the diabetes-induced retinal complication in these dams. Eosinophils are the main regulators of the physiological processes and immune function of perivascular adipose tissue (Withers et al., 2017). According to Maizels and Allen (2011), eosinophils prevent inflammation caused by obesity because it possibly increases the numbers of eosinophils or Th2 cells. This might be explained because the IL-4 and IL-13 secretion signal gamma peroxisome proliferator activated receptor (PPARγ), which, if activated by appropriate lipids, inhibits the expression of genes that promote inflammation (Szanto et al., 2010). Therefore, it is supposed that eosinophilia present in the diabetic and obese group was due to the homeostatic mechanism, tending to minimize the possible inflammation caused by obesity.

The reproductive analysis of the animals in this study showed embryonic losses before and after implantation, which were higher in both diabetic groups, demonstrating the influence of hyperglycemia on the implantation process. Regardless of the degree of severity, hyperglycemia is related to pre- and postimplantation losses in the intrauterine environment (Sinzato et al., 2011; Bequer et al., 2018; Gallego et al., 2018). Moreover, problems with cytokine regulation, which occurs in diabetic pregnancy, can lead to damage during early embryonic development, such as pre-implant failure, leading to a reduced number of implants and postimplantation losses indicated by an increased rate of resorption and a reduced number of live fetuses (Sinzato et al., 2011; Dela Justina et al., 2017). These findings contributed to a lesser weight gain during pregnancy and maternal final weight at term pregnancy. However, obesity did not increase embryo loss rates in the animals.

For the success of pregnancy, it is essential that, during the implantation period, the physiological and molecular processes are coordinated, involving close interactions between the uterus and the blastocyst (Cha et al., 2012). Then, the dams presenting biochemical alterations induced by diabetes, obesity, and both contributed to impaired reproductive outcomes. In relation to fetal development and growth from diabetic and/or obese dams, our study demonstrated that only altered diet caused intrauterine growth restriction, which was confirmed by reduction of the fetal weight, higher percentage of small fetuses for the gestational age, and decline of ossification sites. These findings corroborate other authors since diabetes (Damasceno et al., 2014) and maternal obesity and high-calorie intake (Zou et al., 2017) may impair fetal development. Growth restriction may be related to different maternal adaptations to diet and diet components, with maternal nutrition being a possible factor in intrauterine growth restriction (Howie et al., 2009; Setia and Sridhar, 2009; Mark et al., 2011; Tellechea et al., 2017). The animals that received a high fat/sugar diet had altered placental weights, which may be related to functional or morphological placental

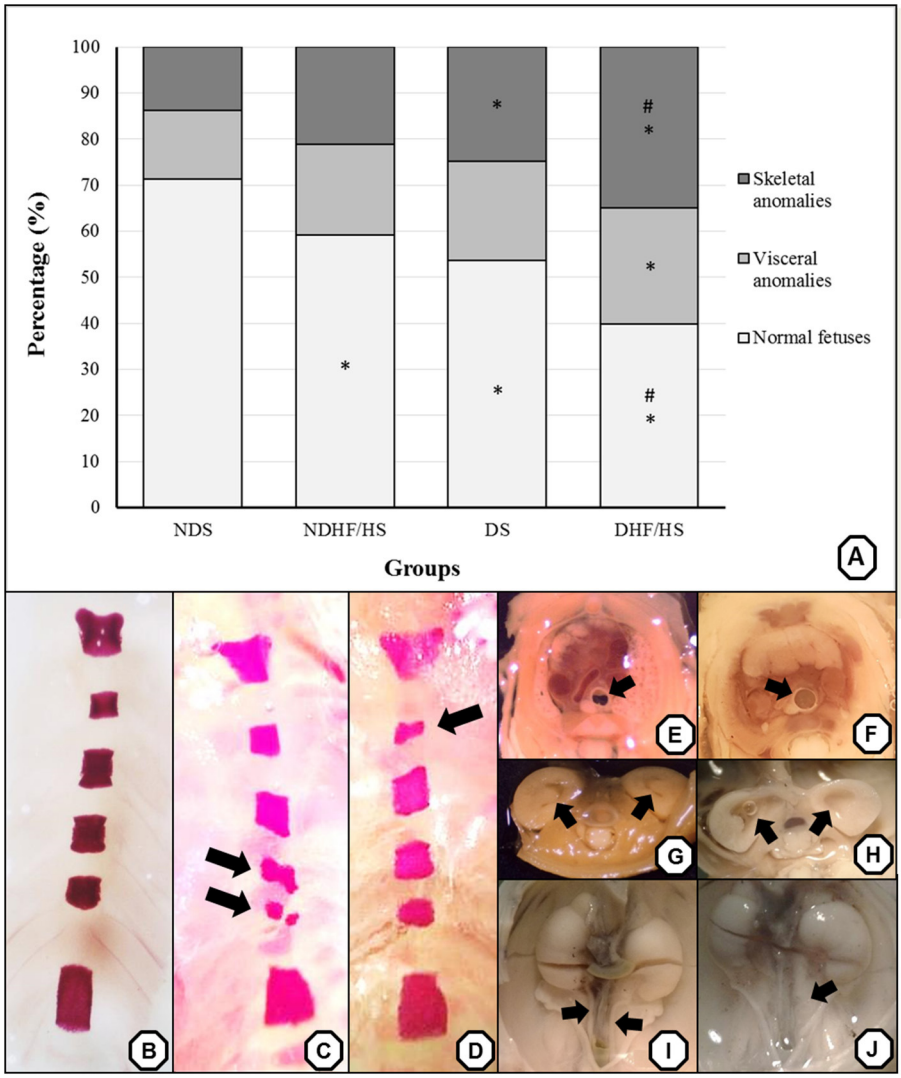

FIGURE 4 | (A) Percentage (%) of anomalies of fetuses from non-diabetic (ND) and diabetic (D) rats in treated or not (S) with high-fat/high-sugar diet (HF/HS) before and during pregnancy. Panels **(B–J)**—representative images of the main skeletal **(B–D)** and visceral **(F–J)** anomalies. Panel **(B)**—normal sternebra of rat fetuses. Panel **(C)**—abnormally shaped sternebra and bipartite ossification of sternebra (arrow). Panel **(D)**—incomplete ossification of sternebra (arrow). Panels **(E,F)**—a thoracic section from rat fetuses, with **(E)** normal trachea (arrow) and **(F)** dilated trachea (arrow). Panels **(G,H)**—a kidney transversal section from rat fetuses, with **(G)** normal renal calices (arrow) and **(H)** dilated renal calices (arrow). Panels **(I,J)**—the pelvis section from rat fetuses, with **(I)** normal ureter (arrow) and **(J)** enlarged ureter—hydroureter (arrow). $^{*}p < 0.05$—compared with the NDS group; $^{\#}p < 0.05$, compared with the NDHF/HS group (Fisher's exact test).

alterations. These placental changes may have contributed to the decrease in fetal weight. Intrauterine growth restriction leads to a variety of phenotypes related to the metabolic syndrome in adult children, including hypertension (Tain et al., 2017; Bendix et al., 2020). In addition, the obesity induced by high-fat/high-sugar diet, whether associated or not associated with diabetes, decreased the frequency of fetuses without anomalies. The diabetes status increased skeletal anomalies. The rats presenting diabetes and obesity showed an exacerbated percentage of fetal abnormalities, with an increased frequency of visceral anomalies. Bueno et al. (2020) already demonstrated that maternal hyperglycemia causes an abnormal fetal metabolism, contributing to an increase of visceral anomalies in the offspring of diabetic rats. Besides, fetal metabolic dysregulation may also occur due to the maternal consumption of a carbohydrate and lipid-rich diet (Musial et al., 2017), which interferes with various pathways of the developing organs, such as the liver, skeletal muscle, adipose tissue, brain, and pancreas (Heerwagen et al., 2010).

This study points out strengths as several variables and biomarkers evaluated, using a solid and structured experimental model; however, it is a study performed in laboratory animals

and needs more attention for human application. For limitation, we have the lack of measurement of leptin, since it could be used during the discussion of appetite and feed intake. Another limiting factor is the determination of the free fat acid levels to relate to other lipid parameters.

In conclusion, the association between maternal diabetes and obesity induces metabolic, leukocyte, and biochemical alterations that contribute to affect fetal development and growth. Further studies are needed to clear more the mechanisms involved during diabesity in pregnancy to prevent the fetal/neonatal outcomes in humans. Then, the experimental model employed in our study helps understand some pathophysiological mechanisms linked to this association, allowing interventionist methods to avoid maternal changes and, consequently, fetal repercussions as found in this study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Ethical Committee for Animal Research of Araguaia—UFMT, Brazil (Protocol number 23108.022251/2019-61).

AUTHOR CONTRIBUTIONS

VA-S and GV: conception and design, data acquisition, data analysis and interpretation, and manuscript writing. AS-S, AL, and CB-B: data acquisition, data analysis and interpretation, and manuscript writing. RM-S, TS, VP, and YS: data interpretation and manuscript writing. BK: manuscript writing. DD: data analysis and interpretation and manuscript writing. All authors have reviewed the manuscript, agreed with its contents, consented to its publication, and that there are no other persons who satisfied the criteria for authorship. We further confirm that all of us have approved the order of the authors listed in the manuscript.

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Circulating MicroRNAs in the Second Trimester From Pregnant Women Who Subsequently Developed Preeclampsia: Potential Candidates as Predictive Biomarkers and Pathway Analysis for Target Genes of miR-204-5p

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MicroRNAs (miRNAs) play an important role in the pathophysiology of preeclampsia (PE). However, the expression of circulating miRNAs was not analyzed in the second trimester of pregnancy, a period of major relevance to identify predictive biomarkers for PE. Therefore, we examined the expression profiles of 84 circulating miRNAs using a PCR array in plasma collected between 20 and 25 weeks of gestation from pregnant women, who subsequently developed PE and those who remained healthy during pregnancy, randomly selected from a prospective cohort. Overall, 23 miRNAs had a fold change > 2.0 and were considered to be upregulated in plasma from pregnant women who subsequently developed PE, even before the onset of clinical symptoms of PE. However, only miR-204-5p was statistically significant ($P = 0.0082$). Experimentally validated interactions for the target genes of miR-204-5p extracted from miRTarBase were used in the gene set functional enrichment analysis to identify Reactome pathways. The network connecting the 37 target genes for miR-204-5p revealed pathways of known pathophysiological relevance during the early development of PE and included key genes related to PE, such as *BDNF, MMP-9*, *MALAT1*, *TGFBR2*, and *SIRT1*. We further depicted downstream targets of SIRT1 that are related to the vascular endothelial function or implicated in the pathophysiology of PE, namely, FOXO1, NFκB, HIF-1α, NOS3, and PPAR-γ. Our novel findings provide for circulating miRNAs upregulated in the second trimester on plasma from pregnant women who subsequently developed PE that is potentially related to the early development of PE, which may guide further studies focused on the validation of potential predictive biomarkers in PE.

Keywords: biomarkers, microRNAs, preeclampsia, pregnancy, gene expression profiling, gene expression regulation, signaling pathways

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INTRODUCTION

Preeclampsia (PE) is characterized by hypertension after 20 weeks of gestation, which may be accompanied by proteinuria or thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema, or cerebral/visual symptoms (American College of Obstetricians Gynecologists Pregnancy TFOHI, 2013). PE affects up to 9% of all pregnancies, it is the major cause of maternal and fetal morbidity and mortality worldwide, and the only definitive treatment is the delivery of the placenta (Umesawa and Kobashi, 2017; Rana et al., 2019). Despite its burden, PE is a multisystem syndrome, and its pathophysiology is complex and not fully elucidated, which limits current management therapies.

MicroRNAs (miRNAs) are short endogenous noncoding RNA transcripts of 18–24 nucleotides that posttranscriptionally regulate gene expression by either degradation or translation repression (Bartel, 2009). It is well known that miRNAs may play an important role in the pathophysiology of PE (Sandrim et al., 2016; Bounds et al., 2017; Caldeira-Dias et al., 2018; Lv et al., 2019; Skalis et al., 2019). Specifically, it has been shown that even before the onset of clinical symptoms of PE, the expression of circulating miRNAs in the first trimester of pregnancy was altered in pregnant women who later developed PE in the third trimester as compared with those who remained healthy during pregnancy (Luque et al., 2014; Hromadnikova et al., 2017, 2019). Since circulating miRNAs are very stable, they have been proved as useful biomarkers for several disorders, including cancer, cardiovascular, and immunoinflammatory diseases (De Guire et al., 2013).

Several studies have focused on identifying specific biomarkers for the early prediction of PE, including miRNAs (Jadli et al., 2015; Lv et al., 2019). To our knowledge, three studies have examined plasma collected in the first trimester of pregnancy to evaluate the deregulated expression of miRNAs as predictors of PE (Luque et al., 2014; Hromadnikova et al., 2017, 2019). The aim to identify biomarkers in the first trimester of pregnancy is to start pharmacological prophylaxis with the use of aspirin and calcium in high-risk women. However, no previous study has analyzed the expression of circulating miRNAs in the second trimester of pregnancy, a period of major importance to identify predictive biomarkers to increase the monitoring of pregnancy, because most of the cases of PE develop after 25 weeks of gestation. Therefore, we aimed to perform a miRNA screening on plasma collected between 20 and 25 weeks of gestation from pregnant women who subsequently developed PE in order to search for differentially expressed miRNAs and provide for potential candidates as predictive biomarkers in PE.

In this study, we examined the expression profiles of 84 circulating miRNAs using a PCR array in plasma collected in the second trimester from pregnant women who subsequently developed PE and compared with those who remained healthy during pregnancy. Moreover, we extracted the target genes for the upregulated miR-204-5p (fold change > 2.4; $P = 0.0082$) from the miRTarBase of experimentally validated miRNA-target interactions and used in the gene set functional enrichment analysis to identify Reactome pathways potentially deregulated in the pathophysiology of PE.

METHODS

Subjects

The study was approved by the Institutional Review Board of the University of São Paulo at Ribeirão Preto (reference 4116/2008), according to the declaration of Helsinki, and all participants provided written informed consent. This case-control study is based in a prospective cohort, Brazilian Ribeirão Preto and São Luís prenatal cohort (BRISA) (da Silva et al., 2014; Pereira et al., 2016; Caldeira-Dias et al., 2019). The schematic diagram of the study workflow is shown in **Figure 1**.

Pregnant women ($n = 1{,}400$) with gestational age between 20 and 25 weeks from our prospective cohort were evaluated at the *Hospital das Clínicas de Ribeirão Preto* at the University of São Paulo (prenatal cohort), but 460 pregnant women gave birth outside the institution. Of the remaining 940 pregnant women, 30 subsequently developed PE and 910 remained healthy during pregnancy (control group). PE was defined as pregnancy-induced hypertension ($\geq$ 140 mmHg systolic and $\geq$ 90 mmHg diastolic on two or more measurements, at least 6 h apart) in a woman after 20 weeks of gestation, and returning to normal by 12 weeks postpartum, and significant proteinuria ($\geq$ 0.3 g/24 h), and it was further classified into mild ($n = 16$) or severe ($n = 14$) according to the American College of Obstetricians and Gynecologists (American College of Obstetricians Gynecologists Pregnancy TFOHI, 2013). Plasma samples from pregnant women with severe PE ($n = 5$, case group) and from the control group ($n = 4$) were randomly selected for this study (**Figure 1**).

Maternal venous blood samples were collected in Vacutainer tubes (Becton-Dickinson, São Paulo, Brazil) using EDTA as an anticoagulant and centrifuged at room temperature. Plasma samples were stored at -80°C until analysis.

Total RNA Isolation and mRNA Expression Using PCR Array

Total RNA from plasma samples (200 μl) from control and case groups was isolated using the miRNeasy Serum/Plasma Kit (Qiagen®, Leusden, Netherlands) according to the instructions of the manufacturer. Noteworthy, we have performed several steps to ensure the quality of the miRNA expression results, which included the calibration of miRNA extraction using cel-miR-39 and the normalization using commonly expressed miRNA targets, as previously reported (Sandrim et al., 2016). Synthetic *Caenorhabditis elegans* cel-miR-39 (#219610, Qiagen®) was added at known amounts during the isolation in each biological sample in order to estimate the efficiency of RNA extraction and reverse transcription (RT) reaction.

The Human Serum & Plasma miScript miRNA PCR Array (MIHS-106Z, Qiagen®, Leusden, Netherlands) was used to evaluate the expression of 84 miRNAs detectable in plasma, as well as seven different small RNAs that were used as normalization control. The PCR array also contained replicate reverse transcription and positive controls.

For the PCR array, the total RNA isolated was converted into cDNA using the miScript II RT Kit (Qiagen®, Leusden, Netherlands), according to the instructions of the manufacturer, which contains a buffer that selectively converts mature miRNAs,

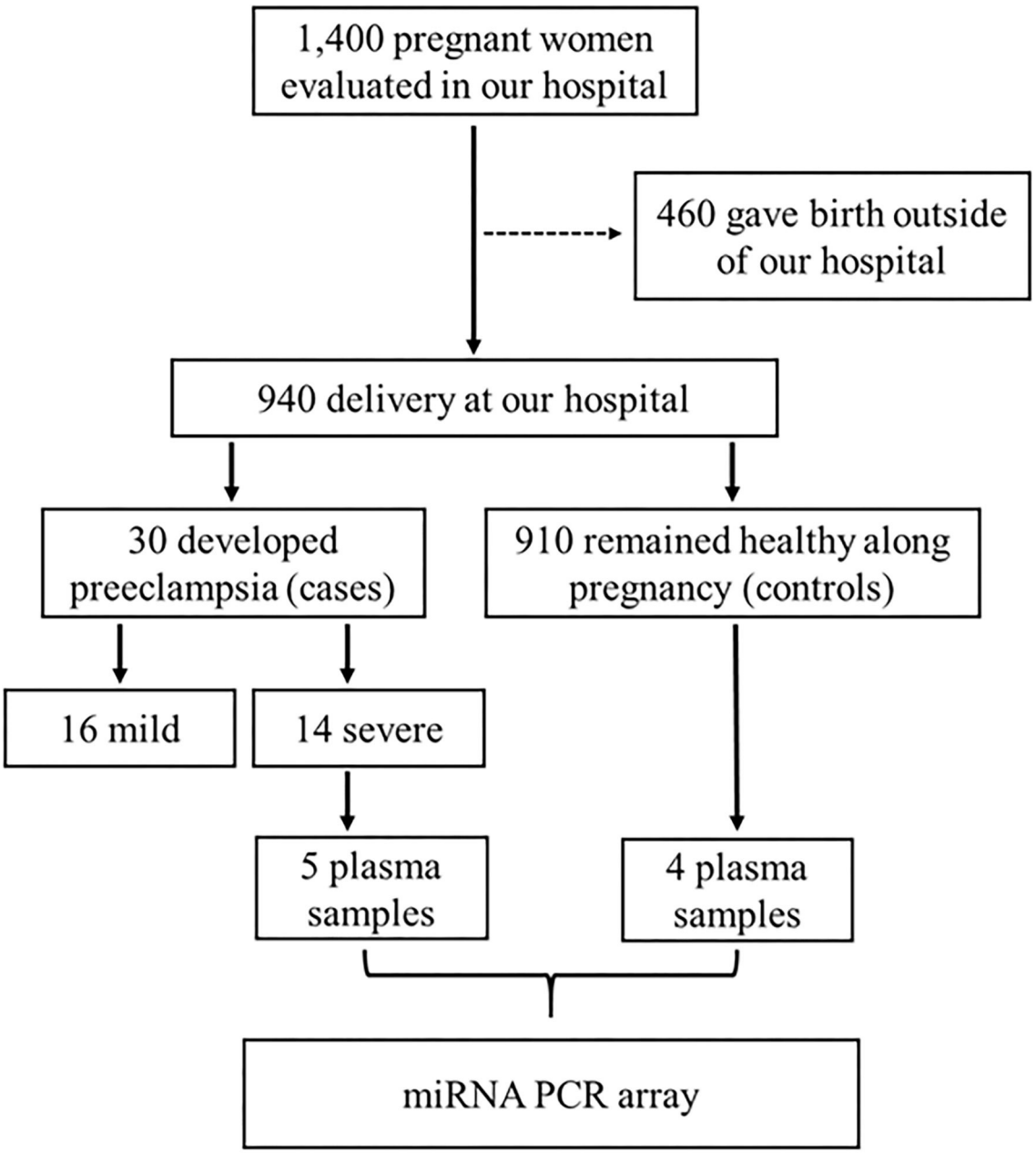

FIGURE 1 | Schematic diagram of the study workflow for the inclusion of subjects, and the selection of plasma samples from pregnant women who developed severe preeclampsia (case group) or those who remained healthy during pregnancy (control group), which were used in the PCR array. This case-control study is based in a prospective cohort named Brazilian Ribeirão Preto and São Luís prenatal cohort (BRISA) (da Silva et al., 2014; Pereira et al., 2016; Caldeira-Dias et al., 2019).

certain small nucleolar RNAs, and small nuclear RNAs into cDNA. qPCR was performed using 1375 μl of 2× QuantiTect SYBR Green PCR Master Mix, 275 μl of 10× miScript Universal Primer, 1000 μl of RNase-free water, and 100 μl of cDNA (1.5 ng/μl) from each sample in a final volume of 2750 μl per plate. Thermal cycling was performed using the following conditions: 15 min at 95°C, 40 two-step cycles of 15 s at 94°C, 30 s at 55°C, 30 s at 70°C, and a final step for the dissociation curve. The C_T (cycle threshold) is the number of cycles required for the fluorescent signal to cross the threshold.

Normalization was performed using the SNORD95, SNORD96A, RNU6-6P, and cel-miR-39 from the housekeeping (HKG) panel in the array, as they were the most stable genes in our samples. The cel-miR-39 was measured concomitantly to the miRNAs of the PCR array. After obtaining the C_T value of each sample, ΔC_T was determined by the equation ΔC_T

$= C_T$sample – Average C_THKG. Then, $\Delta\Delta C_T$ was calculated by the equation $\Delta\Delta C_T = \Delta C_T$PE – ΔC_THP. The fold change in miRNA expression was calculated using the comparative $2(-\Delta\Delta C_T)$ method (Livak and Schmittgen, 2001).

Statistical and miRNA Expression Data Analyses

Clinical characteristics between pregnant women who subsequently developed PE during pregnancy (case group) and those who remained healthy during pregnancy (control group) were compared using the Student's *t*-tests, which were performed using GraphPad Prism 5.0 Software (San Diego, CA, USA).

The QIAGEN's GeneGlobe Data Analysis Center (https://geneglobe.qiagen.com/us/analyze/) online platform was used to analyze the miRNA expression data. Briefly, the CT values for the PCR arrays with individual plasma samples from the case and the control groups were exported to an Excel file and uploaded into the GeneGlobe Data Analysis Center, which calculated the fold change using the $2(-\Delta\Delta C_T)$ method. The *P*-values were calculated based on the Student's *t*-test of the replicate $2^{(-\Delta CT)}$ values for each gene in the case and the control groups. A value of fold change > 2.0 in the case compared with control groups was the cutoff value used to consider the expression of miRNAs as upregulated (Zhao et al., 2018). For all tests, a $P < 0.05$ (two-tailed test) was considered significant.

Experimentally Validated Interactions for Target Genes of miR-204-5p From miRTarBase

Target genes of the upregulated miR-204-5p were extracted from the selection of experimentally validated miRNA-target interactions in *Homo sapiens* included in the miRTarBase database (Huang et al., 2020). The miRTarBase contains manually curated information from research articles that actually performed the experiments to validate the target genes of miR-204-5p, which are referenced by the respective PubMed ID (PMID; **Supplementary Table 1**). Notably, almost all experiments were luciferase reporter assays, along with qRT-PCR, western blot, and/or immunohistochemistry (**Supplementary Table 1**).

Gene Set Functional Enrichment Analysis and Reactome Pathways

Based on the experimentally validated target genes of miR-204-5p from miRTarBase, we then performed the gene set functional enrichment analysis using the enricher function of the R package clusterProfiler (Yu et al., 2012; Chen et al., 2013) in the Reactome Pathway Database (Jassal et al., 2020). Reactome functions both as an archive of biological processes and as a tool for discovering functional relationships in data such as gene expression profiles (Jassal et al., 2020). Pathways that were specific to cancer or specific to other diseases and very general signaling pathways that contained over 200 genes were excluded from the search due to gene overlap and redundancy.

TABLE 1 | Demographic and clinical characteristics of subjects enrolled in the study.

Parameters	Preeclampsia (case group)	Healthy pregnant (control group)	*P*
Number of subjects	5	4	
Age (years)	29.8 ± 2.0	28.8 ± 2.6	0.7541
BMI at sampling (kg/m^2)	28.6 ± 1.8	28.5 ± 0.7	0.9664
SBP (mmHg) at sampling	115.0 ± 5.7	102.5 ± 3.2	0.1199
DBP (mmHg) at sampling	73.0 ± 6.0	62.5 ± 1.4	0.1755
GA at sampling (weeks)	23.4 ± 0.6	22.0 ± 0.6	0.1429
Newborn weight (g)	1942.0 ± 169.4	3623.0 ± 258.4	0.0008*

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; GA, gestational age.
**P < 0.05 vs. control group.*
Data as mean ± S.E.

RESULTS

The demographic and clinical characteristics of pregnant women who subsequently developed PE and those who remained healthy during pregnancy enrolled in the study are shown in (**Table 1**). At the time that maternal venous blood samples were collected, all the demographic and clinical characteristics of the subjects included, such as age, body mass index, systolic and diastolic blood pressure, and gestational age at sampling were similar between the study groups. As expected, the mean newborn weight of pregnant women who subsequently developed PE (case group) was significantly lower than that of the healthy pregnant women included in the control group ($P < 0.05$, **Table 1**).

The expression profile for all the 84 circulating miRNAs assessed in plasma samples from pregnant women who subsequently developed PE (case) and those who remained healthy during pregnancy (control), the fold change values between the groups (case/control), and the *P*-values are shown in **Supplementary Table 2**. Overall, 23 miRNAs were considered to be upregulated by using the fold change > 2.0 in plasma samples from pregnant women who subsequently developed PE as compared to those who remained healthy during pregnancy. However, only the miR-204-5p had a null hypothesis of significance testing lower than 0.05 ($P = 0.0082$; **Table 2**). Notably, the miR-24-3p, miR-22-3p, miR-143-3p, and miR-376c-3p showed a fold change > 4.0, which means that their expression was more than four times higher in pregnant women who subsequently developed PE than that in women who remained healthy during pregnancy.

Noteworthy, we searched the miRTarBase for experimentally validated miRNA-target gene interactions for the miR-204-5p, which were used in the functional enrichment to thoroughly explore the network connecting the 37 target genes and their biological effects (**Figure 2A**). Notably, we found several important genes related to the pathophysiology of PE, such as matrix metallopeptidase 9 (*MMP-9*), metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*), transforming growth factor beta receptor 2 (*TGFBR2*), and sirtuin-1 (*SIRT1*).

TABLE 2 | Fold-change values for the circulating miRNAs expression in plasma from pregnant who developed severe preeclampsia (case group) compared with those who remained healthy during pregnancy (control group).

miRNAs	Fold change (case/control)	*P*
hsa-miR-24-3p	9.4011	0.4951
hsa-miR-22-3p	5.2683	0.2170
hsa-miR-143-3p	5.0049	0.3910
hsa-miR-376c-3p	4.6649	0.2309
hsa-miR-19b-3p	3.2986	0.1323
hsa-miR-17-5p	3.234	0.1492
hsa-miR-106b-5p	3.1587	0.1021
hsa-miR-15a-5p	3.1576	0.1726
hsa-miR-19a-3p	2.8865	0.1779
hsa-miR-29a-3p	2.7242	0.2728
hsa-miR-128-3p	2.7185	0.4609
hsa-miR-200b-3p	2.6691	0.1673
hsa-miR-146a-5p	2.6296	0.2794
hsa-miR-34a-5p	2.4357	0.2510
hsa-miR-204-5p	2.3823	0.0082*
hsa-miR-27a-3p	2.3683	0.4159
hsa-miR-26b-5p	2.3179	0.8274
hsa-miR-223-3p	2.1448	0.8807
hsa-miR-221-3p	2.0681	0.2896
hsa-miR-18a-5p	2.0467	0.2190
hsa-miR-15b-5p	2.0446	0.7782
hsa-miR-103a-3p	2.0249	0.3685
hsa-miR-30e-5p	2.0172	0.6543

A fold change > 2.0 was used as the cutoff value to consider the upregulated expression of miRNAs as compared with the control group.
**P < 0.05 for the miR-204-5p expression in the case group as compared with the control group.*

Noteworthy, *SIRT1* was found to be downregulated by independent analyses in PE (Viana-Mattioli et al., 2020). Moreover, SIRT1 was shown to have a role in regulating endothelial function, arterial remodeling, and vascular aging (Man et al., 2019). Therefore, we depicted downstream targets of SIRT1 that are related to vascular endothelial function or pathways implicated in the pathophysiology of PE (**Figure 2B**).

DISCUSSION

In this study, the main novel findings were that 23 circulating miRNAs were considered to be upregulated by using the fold change > 2.0 in plasma samples collected in the second trimester from pregnant women who subsequently developed PE as compared with those who remained healthy during pregnancy, even before the onset of clinical symptoms of PE. Noteworthy, only the miR-204-5p was statistically significant ($P = 0.0082$).

The search for potential predictive biomarkers is clinically relevant because it enables the early identification of pregnant women at high risk of developing PE, which may allow to take actions to minimize future complications for the mother and fetus. Since most of the cases of PE occur after 25 weeks of gestation, we focused on the period of 20–25 weeks of gestation in order to maximize the chance to find predictive biomarkers to monitor the pregnancy. In this context, several candidates for circulating biomarkers have been studied, such as pregnancy-associated plasma protein-A (PAPP-A), C-reactive protein (CRP), interleukin-1β (IL-1β), antiangiogenic molecules, homocysteine, oxidative stress markers, and vasoactive peptides (Levine et al., 2004; D'antonio et al., 2013; Maged et al., 2017; Rocha-Penha et al., 2017; Lind Malte et al., 2018; Sandrim et al., 2018a,b; Machado et al., 2019). However, most of these biomarkers showed poor to moderate predictive value for PE. Noteworthy, the sFLT-1/PIGF ratio has been shown to be the most promising biomarker, which is the combination of increased levels of the antiangiogenic factor soluble fms-like tyrosine kinase-1 (sFLT-1) along with the decreased levels of placental growth factor (PIGF) (Cerdeira et al., 2019).

The expression profile of circulating miRNAs was reported to be different in pregnant women who subsequently developed PE. However, only some of the miRNAs reported had predictive value for PE (Luque et al., 2014; Hromadnikova et al., 2017, 2019). Notably, miR-143 and miR-221 were found to be upregulated in samples collected in the first trimester (Luque et al., 2014). In this study, we found that 23 miRNAs were considered to have upregulated expression by using the fold change > 2.0 (Zhao et al., 2018) in plasma samples collected in the second trimester from pregnant women who developed PE. Among these, the miR-143-3p, miR-22-3p, and miR-24-3p had a fold change > 4.0 in pregnant women who developed PE, which suggests their role in the early stages of the pathophysiology of PE. Notably, miRNA-143 is essential for the differentiation of vascular smooth muscle cells (Zhao et al., 2015), and its levels were significantly increased in peripheral blood leucocytes from hypertensive patients (Chen et al., 2018). Moreover, miR-22 was found to be upregulated in preeclamptic placentas (Shao et al., 2017), and miR-24 was found to be increased in plasma from patients with severe preeclampsia (Wu et al., 2012). However, only the miR-204-5p was statistically significant ($P = 0.0082$), while the other 22 miRNAs were upregulated but not statistically significant (**Table 2**).

The functional enrichment of target genes for the upregulated miR-204-5p in the Reactome Pathway Database revealed pathways associated with the pathophysiology of PE (**Figure 2A**). Noteworthy, miR-204-5p directly binds to transforming growth factor beta receptor 2 (TGFBR2), a part of the TGF-beta-binding complex. Notably, disruption of TGF-beta signaling is a common characteristic in PE, as reviewed elsewhere (Powe et al., 2011) with excess secretion of soluble endoglin and soluble Flt1 (sFlt-1) directly dysregulating this signaling (Wang et al., 2009). miRNAs could be another player acting to dysregulate TGF-beta-related pathways, which could help to explain the endothelial cell dysfunction and decreased nitric oxide production previously found in severe PE (Powe et al., 2011). Moreover, miR-204 was shown to suppress trophoblast invasion by targeting the MMP-9 (Yu et al., 2015), and it was significantly overexpressed in the preeclamptic placenta (Choi et al., 2013). MALAT1, a long noncoding RNA commonly found to be hyperexpressed in cancer (Tian and Xu, 2015), is also essential for trophoblast invasion

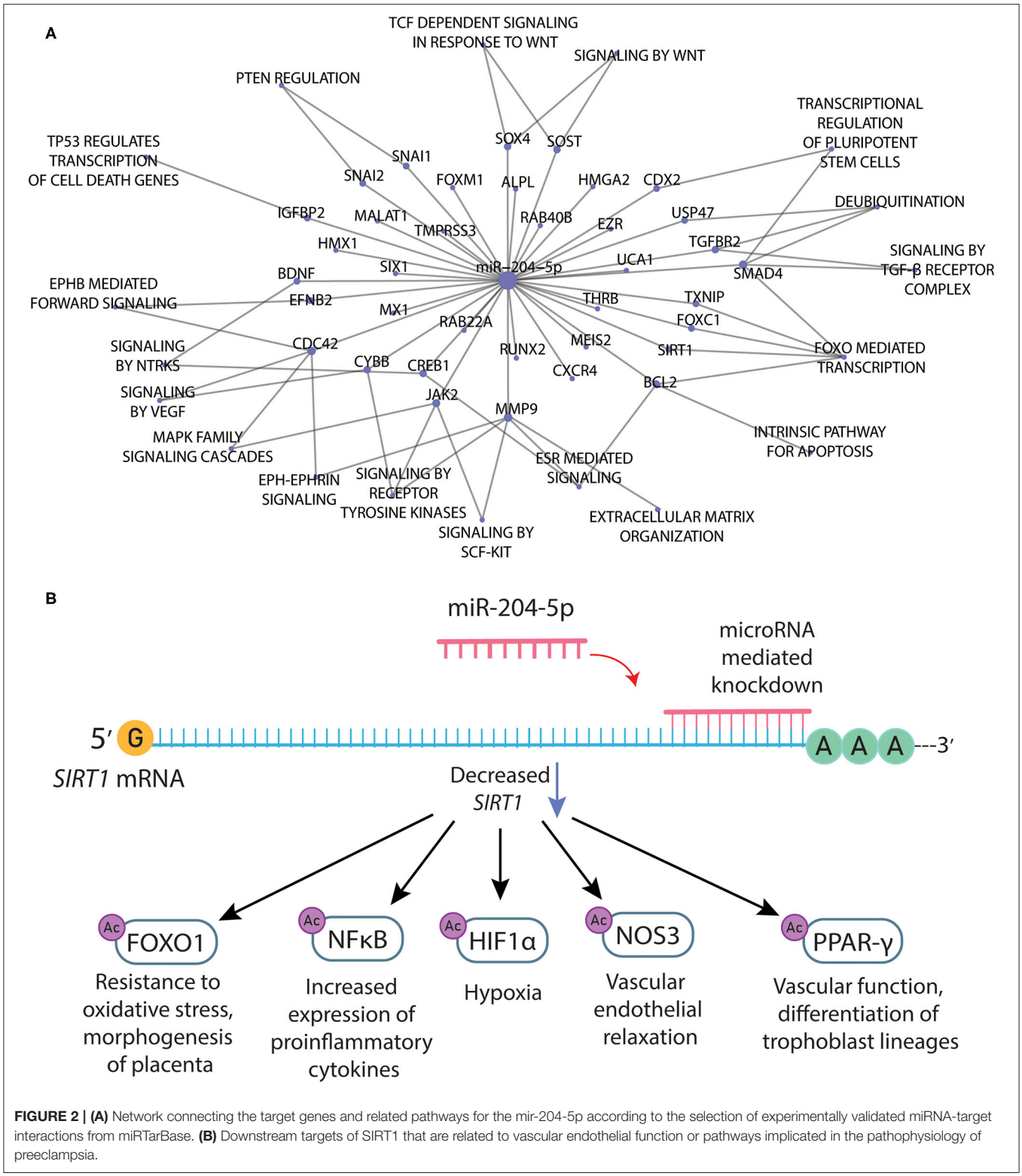

FIGURE 2 | (A) Network connecting the target genes and related pathways for the mir-204-5p according to the selection of experimentally validated miRNA-target interactions from miRTarBase. **(B)** Downstream targets of SIRT1 that are related to vascular endothelial function or pathways implicated in the pathophysiology of preeclampsia.

and proliferation in the early stages of pregnancy (Li et al., 2017, 2020), and it was found to be downregulated in patients with PE. Noteworthy, the miR-204-5p interacts with MALAT1 (Li et al., 2016), which could explain its downregulation in PE patients. However, this hypothesis remains to be tested. Among the target genes for miR-204-5p, there are also several ephrin ligands and

signaling proponents, such as ephrin 2 (*EFNB2*) and cell division cycle 42 (*CDC42*). Notably, ephrin-related pathways have a major role in embryogenesis, and other upregulated miRNAs found in preeclamptic placentas were shown to target related genes (Wang et al., 2012). Taken together, these findings indicate that target genes for the miR-204 could also be involved in the early pathogenesis of PE.

The miRNA 204-5p was found to be upregulated in the serum of patients with PE, and its knockdown induced a better survival rate of hypoxic cells (Mei et al., 2017). However, this previous study did not explore the target genes for the miRNA 204-5p nor the mechanisms of action of these target genes. Interestingly, we found that some of the target genes for the miRNA 204-5p were also found to be downregulated by independent analyses in PE, such as brain-derived neurotrophic factor (*BDNF*) (Perucci et al., 2017) and *SIRT1* (Viana-Mattioli et al., 2020). For example, SIRT1 can deacetylate histones and lead to gene silencing, and it can also regulate the function of several target proteins by deacetylation (Gomes et al., 2016). Endothelial SIRT1 is proven to regulate endothelial function, arterial remodeling, and vascular aging (Man et al., 2019) by interacting with a large network of proteins, such as the forkhead box class O family member proteins (FoxOs), the nuclear factor kappa B (NF-κB), tumor protein 53 (p53), hypoxia-inducible factor-1a (HIF-1α), the superoxide dismutase (SODs), the peroxisome proliferator-activated receptor-γ (PPAR-γ) and its coactivator-1α (PGC-1α) (Chong et al., 2012). Therefore, we depicted selected downstream targets of SIRT1 that have implications for the pathophysiology of PE (**Figure 2B**) and described their biological roles below.

FOXO1 is implicated in the regulation of oxidative stress and apoptosis (Gomes et al., 2016). Moreover, FOXO1 is implicated in the regulation of a variety of other cellular processes that are critical for the placenta, including cell cycle regulation, cellular differentiation and proliferation, DNA repair, and metabolism (Monsalve and Olmos, 2011). Interestingly, FOXO1 expression in the developing mouse embryo was observed to be essential for placental morphogenesis (Ferdous et al., 2011). Since FOXO1 is critical for placental cellular morphogenesis, abnormal FOXO1 expression may contribute in part to the abnormal trophoblast differentiation in mild PE (Sheridan et al., 2015). The NFκB protein family regulates several pathways within the cell-including inflammation, hypoxia, angiogenesis, and oxidative stress, all of which are implicated in placental development (Armistead et al., 2020). Notably, the role of hypoxia and HIF-1α in the pathogenesis of PE, and the possible molecular links between hypoxia and potential mediators of PE pathogenesis are reviewed elsewhere (Tal, 2012). Finally, PPAR-γ plays a predominant role in normal vascular function (Marx et al., 1999) and in the differentiation of trophoblast lineages (Schaiff et al., 2000); it was suggested to play a pivotal role in the progression of a healthy pregnancy and may critically regulate the risk of PE (Mccarthy et al., 2011).

SIRT1 also regulates the endothelial nitric oxide synthase (eNOS), also known as nitric oxide synthase 3 (NOS3), which is activated to produce the vasodilator nitric oxide (NO), upon deacetylation of lysines 496 and 506 in the eNOS calmodulin-binding domain (Mattagajasingh et al., 2007). Noteworthy, previous studies proposed that SIRT1 and eNOS are synergistically regulated through an eNOS-NO-SIRT1 positive feedback mechanism that is considered crucial for maintaining regular endothelium function (Ota et al., 2010; Man et al., 2019). Therefore, reduced SIRT1 may play an important role in PE and was shown to contribute to vascular endothelial dysfunction with aging via modulation of eNOS (Ota et al., 2007; Donato et al., 2011). Interestingly, it was also suggested that SIRT1 activation might be related to reduced sFlt-1 secretion in preeclamptic placentas (Cudmore et al., 2012) and trophoblasts (Hannan et al., 2017; Hastie et al., 2019), thereby reducing the effects of this antiangiogenic molecule and improving vascular dysfunction in PE.

This study has limitations. The power of the study regarding the small sample size may have limited our chance to find statistical differences in the expression of miRNAs. Moreover, we did not perform the validation of the miRNAs considered to be differently expressed in a larger quantity of samples using a different experimental method. Therefore, our findings should be replicated on a larger or secondary population. Importantly, further validation studies are needed to investigate the circulating miRNAs identified in our screening based on a prospective cohort of pregnant women as potential predictive biomarkers in PE. However, since the literature is scarce, our findings provide evidence suggesting that miRNAs found to be upregulated in the second trimester from pregnant women who subsequently developed PE could be prioritized as potential candidates by further validation studies focused on establishing predictive biomarkers in PE.

CONCLUSION

In conclusion, 23 circulating miRNAs had a fold change > 2.0 and were considered to be upregulated in plasma samples collected between 20 and 25 weeks from pregnant women who subsequently developed PE, even before the onset of clinical symptoms of PE. However, only the miR-204-5p was statistically significant ($P = 0.0082$), which has target genes associated with pathways of known pathophysiological relevance during the early development of PE. Therefore, our novel findings provide for circulating miRNAs identified in the second trimester of pregnancy that may guide further studies focused on the validation of potential predictive biomarkers in PE.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a article that does not adhere to our open data policies.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board of the

University of São Paulo (USP) at Ribeirão Preto (reference 4116/2008), State of São Paulo, Brazil, and all participants provided written informed consent. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML, IC, MC-D, RC, and VS have made substantial contributions to the conception of the work. ML, IC, SV-M, MC-D, and VS have drafted the manuscript and prepared the figures. ML, IC, SV-M, MC-D, RC, and VS have revised and approved the final version of the manuscript for submission. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.678184/full#supplementary-material

Supplementary Table 1 | miRTarBase information on the validated target genes for hsa-miR-204-5p, which were verified by functional miRNA–target interactions and experiments (almost all of them by luciferase reporter assays) performed by manually curated articles (referenced by the respective PubMed ID (PMID).

Supplementary Table 2 | Expression profile of 84 circulating miRNAs in plasma samples from pregnant who subsequently developed PE (case group) compared with women who remained healthy during pregnancy (control group). Fold-change values > 2.0 for the upregulated miRNAs in pregnant who subsequently developed PE are highlighted in bold. *miR-204-5p $P < 0.05$ as compared with the expression in the control group.

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REVIEW
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Intrauterine Programming of Cardiovascular Diseases in Maternal Diabetes

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Maternal diabetes is a prevalent pathology that increases the risk of cardiovascular diseases in the offspring, the heart being one of the main target organs affected from the fetal stage until the adult life. Metabolic, pro-oxidant, and proinflammatory alterations in the fetal heart constitute the first steps in the adverse fetal programming of cardiovascular disease in the context of maternal diabetes. This review discusses both human and experimental studies addressing putative mechanisms involved in this fetal programming of heart damage in maternal diabetes. These include cardiac epigenetic changes, alterations in cardiac carbohydrate and lipid metabolism, damaging effects caused by a pro-oxidant and proinflammatory environment, alterations in the cardiac extracellular matrix remodeling, and specific signaling pathways. Putative actions to prevent cardiovascular impairments in the offspring of mothers with diabetes are also discussed.

Keywords: heart, offspring, animal model, humans, diabetes mellitus, intrauterine programming, pathways

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INTRODUCTION

Cardiovascular diseases are increasing at alarming rates in both developed and developing countries (Balakumar et al., 2016; Bhatnagar et al., 2016). Although lifestyle choices and genetic predisposition are the main contributors to cardiometabolic diseases, growing evidence indicates that *in utero* exposure to adverse environmental conditions leads the developing offspring to have numerous risk factors, which may have an impact later in life. The concept of developmental programming was first introduced more than 20 years ago by Dr. David Barker, who investigated the association between low birth weight and increased risk of coronary disease in adult life (Barker, 1998). This phenomenon describes the process by which a stimulus or insult during critical periods of growth and development has lasting effects on the structure or function of tissues and organ systems, which will in turn influence changes in body structure and function permanently. Programming occurs because there is a deregulation of the endocrine or metabolic function, or a failure in the development, growth, or interaction of tissues and organ systems of the body. These alterations involve disruptions in gene expression, cell differentiation, proliferation, communication, and/or signaling during critical periods of fetal life and infancy caused by adverse environmental influences. In particular, the risk of fetal programming of metabolic and cardiovascular diseases is well-known to be increased by diabetes in pregnancy (Pandey et al., 2015; Lohse et al., 2018).

Diabetes can be diagnosed before pregnancy (pregestational type 1 or type 2 diabetes) or have its onset during pregnancy (gestational diabetes mellitus, GDM). All types of diabetes have been shown to induce an adverse fetal programming (Poston, 2010). Epidemiological studies performed in both developed and developing countries have shown an increase in the prevalence and incidence of type 2 diabetes and GDM over the years (Trujillo et al., 2015; Zhu and Zhang, 2016; Hills et al., 2018; Najafi et al., 2019; Gorban de Lapertosa et al., 2021). This review provides information on studies of both human and experimental models addressing putative mechanisms involved in the fetal programming of heart damage in maternal diabetes. Shedding light on these mechanisms would provide tools for future studies in the prevention of cardiovascular disease in offspring exposed to maternal diabetes.

HUMAN STUDIES

Several studies have shown that children and adolescents exposed to maternal diabetes *in utero* have significantly higher systolic and mean arterial blood pressures (Cho et al., 2000; Lu et al., 2019; Pathirana et al., 2019). In addition, a correlation has been found between GDM and the rate of cardiovascular disease-related hospitalizations of the offspring up to 18 years (Leybovitz-Haleluya et al., 2018). A recent study has shown that fetuses from diabetic gestation present an increase in the left ventricular mass and wall thickness, which persist into late infancy (Do et al., 2019). The follow up of this longitudinal study in childhood has shown that these alterations persist, with the offspring from diabetic mothers showing higher aortic and ventricular stiffness (Do et al., 2021). Further, a 40-year follow-up study in Denmark has shown that offspring of mothers with diabetes have 29% increased overall rate of early onset of cardiovascular heart disease (heart failure, hypertensive disease, deep vein thrombosis, and pulmonary embolism; Yu et al., 2019).

Cardiovascular diseases associated with inflammatory processes, as the ones related to diabetes, show increased endothelial cell signal transductions, which favor leukocyte migration by endothelial cell adhesion molecules (Endemann and Schiffrin, 2004). Among these molecules, vascular cell adhesion molecule-1 (VCAM-1) plays important roles in the embryonic development of the cardiovascular system and in cardiovascular diseases (Cook-Mills et al., 2011). In this regards, it has been found that children born to mothers with diabetes during pregnancy have increased levels of VCAM-1 and other markers of endothelial activation like E-selectin (Manderson et al., 2002; West et al., 2011). A study of 21 healthy non-Hispanic White children between 8 and 12 years old exposed to GDM *in utero* reported that, in addition to higher levels of VCAM-1, these children showed an increase in cardiometabolic risk factors, including higher body mass index *z* scores, higher waist circumference and higher levels of triglycerides, low-density lipoprotein-cholesterol, and leptin compared to unexposed children (West et al., 2011). The latter is important because increased levels of leptin may stimulate oxidative stress, inflammatory reactions, atherogenesis, and thrombosis and thus promote endothelial dysfunction, arterial stiffness, and development and vulnerability of atherosclerotic plaques (Katsiki et al., 2018).

Regarding proposed mechanisms of programming of cardiovascular alterations by maternal diabetes, the main protagonist is epigenetics. Epigenetics can be defined as heritable changes that modify gene expression without altering the DNA sequence itself. Epigenomes are sensitive to their environment and are affected by specific environmental cues, being a plausible mechanism that conditions developmental origin of health and disease in later life. Epigenetic responses during critical windows of fetal development alter gene expression in specific organs or tissues, conditioning their developing and immature functionality and resulting in changes in mature tissues and organs. These responses can either protect the organism from or predispose the organism to disease development in later life. Epigenetic modifications include DNA methylation. DNA methylation involves the covalent addition of a methyl group to the cytosine base in CpG dinucleotide islands of regulatory sites of gene promoter regions that regulate gene expression. This pattern of DNA methylation is highly susceptible to abnormal modifications during adverse gestation and neonatal development (Zhou et al., 2011). In this regard, a genome-wide methylation analysis performed in DNA obtained from peripheral blood mononuclear cells from healthy non-Hispanic White children exposed to maternal GDM during intrauterine life showed 84 genes with differentially methylated regions (West et al., 2013). This study also showed that several of the top 10 genes ranked by statistical significance, such as *Natriuretic Peptide Receptor* 1 (*NPR1*; related to blood pressure homeostasis), *panthothenate kinase* (*PANK1*; a critical enzyme in the synthesis of coenzyme A), *SCAN domain-containing protein 1* (*SCAND1*; which encodes a cofactor that interacts with transcription factor regulators of genes involved in lipid metabolism), and *GJA4* (which encodes connexin 37, a component of gap junction channels involved in intercellular communication) were associated with cardiovascular risk (West et al., 2013). Alterations in these genes have been found to be related to hypertensive diseases, altered glucose and lipid metabolism, and myocardial physiology and may thus be related to adverse programming of cardiovascular disease in offspring exposed to maternal diabetes (Babb and Bowen, 2003; Pitzalis et al., 2003; Leu et al., 2011).

Other molecular epigenetic mechanisms that may play a key role in epigenetic inheritance and aberrant development of cardiovascular disease in later life include histone modifications

Abbreviations: VCAM-1, Vascular cell adhesion molecule-1; GDM, Gestational diabetes mellitus; HUVEC, Human umbilical vein endothelial cells; EZH2β, Enhancer of zester homolog 2 – β; STZ, Streptozotocin; *Hspa1a*, *Heat shock protein 1a1*; NPR1, Natriuretic Peptide Receptor 1; PANK1, Panthothenate kinase; SCAND1, SCAN domain-containing protein 1; Sirt1, Silent information regulator 1; FoxO1, Forkhead box protein O1; FGF, Fibroblast growth factor; GSK3β, Glycogen synthase kinase 3 β; PGC1α, Peroxisome proliferator-activated receptor gamma coactivator alpha; mTOR, Mechanistic target of rapamycin; SGK1, Serum- and glucocorticoid-inducible kinase 1; PPAR, Peroxisome proliferator activated receptor; NOX, NADPH oxidases; TBARS, Thiobarbituric acid reactive substances; ECM, Extracellular matrix; TGFβ-1, Transforming growth factor β-1; CTGF, Connective tissue growth factor.

and small non-coding RNAs, including microRNAs (also called miRNAs; Watson et al., 2019).

For example, a study of whole peripheral blood from children aged 3–11 years descending from GDM pregnancies showed that these children present an altered miRNA expression profile (Hromadnikova et al., 2020). The study also showed that a large group of the genes affected by the altered miRNA expression is involved in ontologies of diabetes/cardiovascular/cerebrovascular diseases (Hromadnikova et al., 2020). Moreover, clinical examination of these children indicated that they present an increased incidence of valve problems or heart defects (Hromadnikova et al., 2020).

Studies in human umbilical vein endothelial cells (HUVECs) are an excellent model for the study of vascular endothelium properties and the main biological pathways involved in endothelium function.

Studies in primary culture of HUVECs and human placental microvascular endothelial cells from GDM pregnancies have shown the dysregulation of adenosine metabolism (Westermeier et al., 2011, 2015). Adenosine metabolism, which plays an important role in the methylation cycle, is a proposed mechanism of epigenetic regulation in endothelial cells involved in the cardio-metabolic fetal programming by GDM (Silva et al., 2020).

Another study performed in HUVECs from GDM pregnancies reported a reduction in the expression of histone methyltransferase *enhancer of zester homolog 2 – β* (*EZH2β*) related to an increase in the expression of miR-101, a microRNA that targets *EZH2β* (Kuzmichev et al., 2002). *EZH2β* is part of a multisubunit complex that initiates and maintains the trimethylation of histone H3 on lysine 27 (H3K27me3), an epigenetic mark associated with heterochromatin formation and gene silencing (Cao et al., 2002), and thus probably related to the regulation of this epigenetic mark in endothelial cells from GDM offspring.

The knowledge regarding the mechanisms of programming of cardiovascular disease in offspring from human patients with diabetes is limited because of the difficulty in performing human intervention studies. Thus, the studies of maternal diabetes in animal models are relevant as they allow us to better understand the developmental origins of disease, moving our understanding from associative studies to mechanistic insights into disease causation.

ANIMAL STUDIES

Among animal studies, those performed in rodent models are useful because the order and timing of fetal organ development are similar to those in humans. Moreover, the ability to control various aspects of pregnancy and the pre- and post-natal environment makes rodent models as attractive experimental systems to examine how maternal diabetes during pregnancy programs the physiology of organ systems and increases the risk of cardiometabolic disease in the offspring.

Experimental models of diabetes in pregnancy can have a genetic origin or be obtained by chemical induction with drugs such as streptozotocin (STZ), which, at the appropriate dose, acts by selectively destroying pancreatic cells, leading to insulin deficiency and hyperglycemia in different animals (Jawerbaum and White, 2010).

Regarding the mechanisms underlying the programming of cardiovascular diseases, studies in animal models can be divided into those focused on (1) epigenetic mechanisms, (2) altered pathways of cellular metabolism, (3) alterations induced by a pro-oxidant/proinflammatory environment, and (4) alterations in extracellular matrix remodeling and intercellular communication.

Animal Studies Focused on Cardiovascular Epigenetic Mechanisms

Animal studies focused on epigenetic mechanisms may improve our understanding of how epigenetic gene transcriptional regulation responds to an altered gestational milieu to influence the developmental origin of health and disease.

As an example, a study of the neonatal heart in a mouse model of diabetes showed a 10-fold increase in the DNA methylation of gene promoter regions of many important cardiac genes (Lister et al., 2013). In another study, rat neonatal hearts exposed to maternal diabetes showed altered gene-activating (H3Ac, H3K4me3) and gene-suppressive (H3K27me3) histone marks (Upadhyaya et al., 2017). In this study, chromatin-immunoprecipitation-sequencing and bioinformatics identified that the promoters of two functionally related genes, *heat shock protein 1a1* (*Hspa1a*) and *Hspa1b*, showed enriched H3K4me3 peaks (Upadhyaya et al., 2017). Hspa1a has been proposed as an independent prognostic marker of heart failure (Jenei et al., 2013). This suggests that Hspa1a may be a relevant marker linking maternal diabetes and offspring heart failure.

In a rat model of diabetes in pregnancy induced by STZ administration on day 12 of gestation, cardiomyocytes from 6-week-old offspring showed an increase in global DNA methylation status, together with an increase in the cardiac expression of DNA methyltransferase 3A and reduced expression of the silent information regulator 1 (Sirt1), a class III histone deacetylase (Chen et al., 2019). This altered expression of Sirt1 was reversed *ex vivo* with a DNA-methylation inhibitor and *in vivo* with the antioxidant N-acetyl-cysteine, suggesting that Sirt1 expression in the heart of offspring from dams with diabetes is epigenetically regulated and affected by an oxidative environment (Chen et al., 2019). Sirt1 functions as a regulator of acetylation, which is important to maintain cardiac mitochondrial integrity and normal myocardium development (Planavila et al., 2012). One of Sirt1 targets genes is the transcription factor forkhead box protein O1 (FoxO1), which, in the cardiovascular system, participates in myocardial metabolic stress adaptation, oxidative stress, endothelial dysfunction, and other processes related to inflammation and apoptosis (Kandula et al., 2016). FoxO1 deacetylation by Sirt1 induces the attenuation of its ability to bind DNA and favors its phosphorylation and consequently its inactivation. In line with this, in a rat model of mild diabetes, the heart of male adult offspring was found to show an increase in active FoxO1 (Musikant et al., 2019).

Another putative epigenetic mechanism involved in the programming of cardiovascular diseases involves biogenesis of miRNAs which can be delivered by exosomes. Exosomes are nanovesicles that contain intact and functional mRNAs, miRNAs and proteins, which function as pivotal mediators of cell-cell communication among neighboring or distant cells and play a key role in multiple physiological or pathological processes (Greening et al., 2017).

It has been demonstrated that, during pregnancy, both feto-maternal and maternal-fetal exosomal trafficking can occur, and that exosomes are able to cross placental barriers and reach fetal tissues (Sheller-Miller et al., 2019; Czernek and Düchler, 2020). Although the precise mechanism has not yet been completely clarified, *in vitro* studies have shown that endothelial cells absorb placental-derived exosomes through endocytosis (Tong et al., 2017). A study by Shi et al. of serum exosomes isolated from pregnant mice with STZ-induced diabetes showed that these vesicles present differences in the expression of more than 200 miRNAs (Shi et al., 2017), some of which, including miR-133, miR-30, miR-99, and miR-23, are involved in cardiac development and cardiovascular diseases (Bang et al., 2012; Izarra et al., 2017; Yin and Yang, 2019). These authors also found that serum exosomes isolated from these diabetic pregnant mice, fluorescently labeled for tracking and then injected into normal pregnant mice *via* the tail vein, were able to cross the placental barrier and infiltrate into embryonic tissues, including the heart (Shi et al., 2017). Finally, these authors showed that the injections of these exosomes increased embryonic heart malformations and induced alterations in the cardiac systolic function in the morphologically normal fetus (Shi et al., 2017), highlighting the role of maternal exosome-derived microRNAs in the induction of fetal cardiovascular defects.

Animal Studies Focused on Altered Pathways of Cardiac Cellular Metabolism

Key pathways of cellular metabolism have been shown to be altered in cardiac cells of offspring from animal models of diabetes. A transcriptomic study performed in neonatal heart from dams treated with a combination of high-fat diet and STZ at day 14 of pregnancy, showed many changes in key genes of metabolic cardiac pathways. Among them, a downregulation of *fibroblast growth factor* (*FGF*), which in turn downregulates PI3K/AKT pathway activation that lead to increased *glycogen synthase kinase 3 β* (*GSK3β*) and peroxisome *proliferator-activated receptor gamma coactivator alpha* (*PGC1α*; Preston et al., 2020). PI3K/AKT is an insulin sensitive pathway that modulates metabolic pathways as gluconeogenesis (modulated by GSK3β) and mitochondrial function and biogenesis (modulated by PGC1α), between others. These changes would contribute to an increased translation of mitochondrial proteins, mitochondrial biogenesis, and gluconeogenesis.

Another important pathway of cellular metabolism is regulated by the mechanistic target of rapamycin (mTOR), a cellular sensor for energy metabolism and nutrient availability that controls cellular growth and metabolism. mTOR forms two different complexes (mTORC1 and mTORC2), which differ in downstream signaling pathways and function (Saxton and Sabatini, 2017). Activated mTORC1 plays a central role in the control of cell growth and proliferation, while mTORC2 plays a key role in cell survival, metabolism, proliferation, and cytoskeleton organization (Saxton and Sabatini, 2017). In a study of a rat model of STZ-induced mild diabetes, the heart of adult offspring showed a reduction in mTOR protein levels and in the phosphorylation of serum- and glucocorticoid-inducible kinase 1 (SGK1), a downstream target of the mTORC2 pathway, which phosphorylates FoxO1 (Musikant et al., 2019). Phosphorylation of the FoxO family by SGK1 induces its nuclear exclusion and inactivation, providing another possible mechanism explaining FoxO1 increased activity (Brunet et al., 2001; Musikant et al., 2019). Interestingly, in a study in which a diet enriched in fish oil was administered to dams with STZ-induced diabetes, the offspring's neonatal heart showed an increase in the reduced phosphorylation of mTOR and AKT, both in Ser-473 (phosphorylation induced by mTORC2) and in Thr-308 (phosphorylation activated by growth factors pathways; Nasu-Kawaharada et al., 2013).

In the offspring heart of an obesity model with hyperglycemia and hyperinsulinemia, Zhang et al. found that the mTORC1 signaling pathway was increased. These authors also found that a maternal treatment with the antioxidant N-acetyl-cysteine during pregnancy normalized the altered mTOR signaling in the offspring heart (Zhang et al., 2021).

Regarding lipid metabolism, in maternal diabetes, insulin concentration, and/or signaling deficiency may lead to an excess of metabolic substrates and impairments in maternal lipid metabolism. In addition, diabetes in pregnancy frequently induces dyslipemia in the mother (Herrera and Ortega-Senovilla, 2010). We have previously reviewed the importance of lipids in the programming of metabolic and cardiovascular alterations in the offspring (Higa and Jawerbaum, 2013).

In an experimental model of high-fat and high-cholesterol diet during pregnancy and lactation, which induces dyslipemia, glucose intolerance, and reduced insulin sensitivity, male offspring showed increased blood pressure, impaired vascular reactivity, and lower response to endothelium-dependent vasorelaxation (Guimarães et al., 2020). Interestingly, in that study, an administration of a specific strain of the probiotic *Lactiplantibacillus plantarum* improved the lipid profile and insulin resistance and restored the dysbiotic gut microbiota in dams, reducing blood pressure and recovering the vascular function in the adult offspring (Guimarães et al., 2020). In another study in a STZ-induced experimental model of mild diabetes, 5-month-old offspring showed increased glycemia, insulinemia, and triglyceridemia (Capobianco et al., 2015). Besides, a sex-dependent lipid accumulation was observed in their heart as female offspring showed increased levels of free fatty acids, cholesterol, and phospholipids and male offspring showed increased levels of triglycerides (Capobianco et al., 2015). These results are relevant because an increase in lipid accumulation in the heart has been related to impaired mitochondrial metabolism and dynamics and increased oxidative stress, cardiomyocyte apoptosis, myocardial fibrosis, and contractile dysfunction (McGavock et al., 2006; Wende and Abel, 2010; Elezaby et al., 2015). Although the mechanisms

of sex-dependent lipid overaccumulation in the heart are unknown, they may be due to changes in lipid metabolism, which are in turn dependent on multiple factors, including the sex-dependent impact of hormones, mitochondrial function, and genetic and epigenetic differences (Gabory et al., 2009; Vijay et al., 2015; Regitz-Zagrosek and Kararigas, 2017).

The intrauterine programming of cardiovascular impairments and the regulation of peroxisome proliferator activated receptor (PPAR)-dependent pathways have also been found to be sex-dependent (Benz et al., 2012; Regitz-Zagrosek and Kararigas, 2017). In experimental models of STZ-induced diabetes, PPARα, a nuclear receptor highly involved in heart metabolic processes and lipid metabolism, has been found increased in the heart of both males and females at the neonatal stage but only in that of males at the prepubertal stage (Higa et al., 2017). An increased level of PPARα is a marker of metabolic substrate utilization as this nuclear receptor regulates multiple genes of lipid catabolism (Lefebvre et al., 2006). Oleic acid, present at high concentrations in olive oil, is a natural agonist that interacts with the ligand-binding domain of the PPAR and leads to its activation (Xu et al., 1999). Interestingly, a maternal olive oil-supplemented diet administered during diabetic rat pregnancy prevents lipid overaccumulation in the offspring's heart, probably as a consequence of improving intrauterine metabolic homeostasis (Capobianco et al., 2015). The PPARα gene was susceptible to epigenetic modification in the livers of offspring of dams fed a low-protein diet (Lillycrop et al., 2005) and PPAR expression in the livers of offspring from an animal model of gestational diabetes was found to be modulated by different miRNAs (Fornes et al., 2018). However, further research in needed to elucidate whether maternal diabetes also induces epigenetic modifications of the PPARα gene in the offspring's heart.

In an animal model of diabetes induced by STZ administration during pregnancy, results showed diastolic dysfunction in the offspring at the neonatal stage (Mdaki et al., 2016) and impaired mitochondrial dynamics in neonatal cardiomyocytes (Larsen et al., 2019). The term "mitochondrial dynamics" refers to the coordinated cycles of fission and fusion occurring in these organelles, which are important to maintain their shape, distribution and size. Mitochondrial dynamics plays an important role in both cardiac development and long-term heart health (Dorn et al., 2015). Interestingly, a study performed in cardiomyocytes of neonates from rats with diabetes showed 50% reduction in fusion events and 30% reduction in fission events, as well as a pro-fission imbalance in the ratio of these events and a higher number of fragmented mitochondrial morphology (Larsen et al., 2019).

Alterations in mitochondrial function lead to an increase in reactive oxygen species and thus to an increase in oxidative stress (Peoples et al., 2019), which in turn plays a key role in the programming of cardiovascular alterations (Gupta et al., 2004; Rueda-Clausen et al., 2012).

Animal Studies Focused on Cardiovascular Alterations Induced by a Pro-oxidant/ Proinflammatory Environment

In cardiac tissues, a major source of reactive oxygen species under both physiological and pathological conditions is NADPH oxidases (NOX; Lassegue et al., 2012). In a study performed in a rat model of diabetes in pregnancy induced by STZ administration on day 12 of gestation, the heart of the 6-week-old offspring showed an increase in NOX1 and NOX2 together with an increase in reactive oxygen species in the left ventricle (Chen et al., 2019), and the heart of adult offspring still showed an increase in NOX2 expression (Zhang et al., 2018). Maternal treatment with N-acetyl-cysteine prevents the increased NOX2 transcript levels in the offspring heart from a model of obesity and diabetes (Zhang et al., 2021).

In another study in a model of mild diabetes obtained by STZ administration, the offspring's heart at neonatal stage showed an increase in nitrated proteins, which evidences protein damage by peroxynitrites (Higa et al., 2017), potent oxidant agents generated as the product of nitric oxide and superoxide anion reaction. In that study, neonatal glycemia of offspring exposed or not to maternal diabetes was similar, indicating that peroxynitrite-induced damage in the offspring's heart occurs in the absence of alterations in neonatal glycemia. However, at a prepubertal stage, glycemia was increased only in male offspring from rats with diabetes (Higa et al., 2017).

Lipids can also be the target of reactive oxygen species. In an experimental model of STZ-induced diabetes, sex-dependent differences were found related to lipoperoxidation, as only neonatal hearts of male offspring showed an increase in thiobarbituric acid reactive substances (TBARS), which are formed as a byproduct of lipid peroxidation (Higa et al., 2017). At the fetal stage, lipoperoxidation has also been observed only in the hearts of male fetuses, suggesting changes during fetal development that persist into adult life (Kurtz et al., 2014).

Regarding maternal treatments that have an impact in modulating the pro-oxidant intrauterine environment, at our lab, we observed that a maternal treatment with an olive oil-supplemented diet administered during pregnancy was able to prevent the increased pro-oxidant markers as well as apoptosis in the heart of 21-day-old offspring of diabetic rats (Roberti et al., 2020). At our laboratory, we have also evaluated a maternal treatment with the mitochondrial antioxidant idebenone in a model of mild diabetes during pregnancy to address the role of oxidative stress and mitochondrial dysfunction in the programming of cardiac alterations and observed that this treatment prevents the increase in markers of oxidative damage in the heart of offspring from diabetic rats at a prepubertal stage (Higa et al., 2017). These results highlight the relevance of mitochondrial-related reactive oxygen species in the programming of heart alterations by maternal diabetes.

Animal Studies Focused on Alterations in the Cardiac Extracellular Matrix and Intercellular Communication

The cardiac extracellular matrix (ECM) is a complex and dynamic structure that forms a three-dimensional network in the cardiac interstitium. It provides structural support for several distinct cell types, contains growth factors and cellular adhesion proteins, and integrates extracellular signals and

cellular responses (Frangogiannis, 2017). In cardiac pathological conditions, ECM synthesis and chemical composition are subject to changes under different environmental stimuli that distort the architecture of the matrix network, modulating the proliferation, migration and activation of cardiac fibroblasts, thus playing a major role in the development of cardiac diseases (Li et al., 2018). During organogenesis, mouse embryo cultures in hyperglycemic conditions have been found to show an increased expression of cardiac fibronectin and transforming growth factor β-1 (TGFβ-1; Smoak, 2004), a fibrogenic growth factor that mediates ECM remodeling. In the male offspring heart from a mouse model of obesity and diabetes, Zhang et al. found an increased expression of TGFβ-1 together with myocardial fibrosis and left ventricular structural alterations. These authors also found that all these programmed alterations were prevented by a maternal treatment with N-acetyl-cysteine during pregnancy (Zhang et al., 2021). Several downstream actions of TGF-β1 can be mediated by connective tissue growth factor (CTGF), a secreted multifunctional protein also involved in the regulation of ECM deposition during development as well as during pathological conditions (Frangogiannis, 2012). A study in an animal model of STZ-induced mild diabetes showed an increase in CTGF levels in the hearts of neonates (Higa et al., 2017). In the heart, CTGF has been found to be epigenetically regulated by microRNA-133 and -30 (Duisters et al., 2009), pointing to these miRNAs as putative epigenetic marks that may be involved in the intrauterine programming of CTGF upregulation in the offspring's heart as they have also been found increased in serum exosomes isolated from pregnant dams with diabetes (Shi et al., 2017).

The balance between ECM synthesis and degradation is of crucial relevance in maintaining cardiac structural integrity. In this context, it is important to mention the role of matrix metalloproteinases (MMPs), which are proteolytic enzymes able to degrade ECM components. Expression and/or activity of MMPs are upregulated by the pro-oxidant/proinflammatory environment. In particular, MMP-9 expression has been found

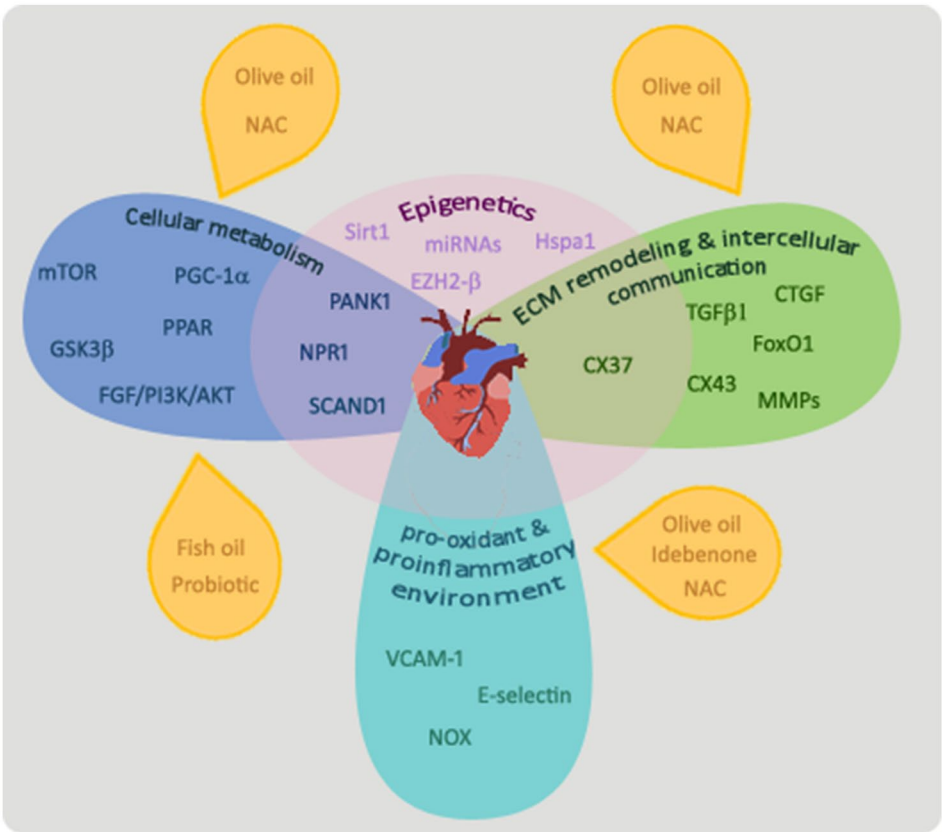

FIGURE 1 | Mechanisms involved in the programming of cardiovascular alterations in offspring by maternal diabetes. Main players that regulate cellular metabolism in the heart of offspring exposed to diabetes *in utero* are mTOR, PPAR, PANK1, PGC1α, GDK3β, NPR1, SCAND1, and the FGF/PI3K/AKT pathway. Maternal treatments with probiotics or a diet enriched in olive or fish oil or N-acetyl-cysteine have been shown to ameliorate cardiac cell metabolism of offspring from diabetic dams. TGFβ-1, CTGF, FoxO1, matrix metalloproteinases (MMPs), and connexin 37 (CX37) and 43 (CX43) have been shown to play important roles in the process of ECM remodeling and intercellular communication. A maternal treatment with N-acetyl-cysteine (NAC) or with an olive oil-supplemented diet was able to prevent alterations in the ECM remodeling process observed in the offspring's heart of animal models of diabetes. An increased pro-oxidant/proinflammatory process and related molecules (VCAM-1, E-selectin, and NOX) have been shown to be involved in the cardiovascular alterations programmed by maternal diabetes. Maternal treatments with an olive oil-supplemented diet or with antioxidants, as idebenone or N-acetyl-cysteine have beneficial effects on this process. All these pathways are interconnected and the underlying mechanism affecting them may be epigenetic phenomena.

increased in hearts of neonates from diabetic rats (Higa et al., 2017). Interestingly, alterations in these markers of ECM remodeling persist and are sex-dependent at a prepubertal stage of development. At 21 days old, only males show an increased expression of MMP-9, possibly related to increased markers of pro-oxidant/proinflammatory processes, which are observed only in the heart of male fetuses and 21-day-old male offspring (Kurtz et al., 2014; Higa et al., 2017; Roberti et al., 2020). Indeed, the stimulation of several fibrogenic pathways by diabetes is related to the generation of reactive oxygen species and the induction of secretion of pro-inflammatory cytokines and chemokines (Tuleta and Frangogiannis, 2021). Moreover, maternal treatment with an olive oil-supplemented diet administered during pregnancy, which has been found to prevent cardiac pro-oxidant/proinflammatory processes in the offspring, is also able to prevent the increased expression of CTGF and deposition of collagen IV and fibronectin in the heart of 21-day-old offspring of diabetic rats (Roberti et al., 2020).

FoxO1 is a TGFβ-1 downstream crucial player in cardiac fibroblast conversion into cardiac myofibroblasts, which, under pathological conditions such as diabetes mellitus, synthesizes and secretes high amounts of ECM proteins (Vivar et al., 2021). Male adult offspring from rats with mild diabetes show an increase in cardiac active FoxO1, together with an increase in the mRNA levels of its target genes in the heart, *Mmp-2* and *Ctgf*, and in collagen deposition and fibrosis (Musikant et al., 2019). These offspring's hearts also show reduced expression of connexin 43, a target of matrix metalloproteinase 2 and main component of the gap junctions and hemichannels in myocytes (Musikant et al., 2019). As connexin 43 is a key factor in electrical coupling and its altered expression alters normal impulse propagation (van Rijen et al., 2006), its reduced levels in the offspring's heart is likely related to cardiac dysfunction. These alterations were found concomitantly with increased markers of cardiomyopathy and increased cardiovascular risk factors as glycemia, triglyceridemia, and insulinemia (Musikant et al., 2019). Together, these results suggest an important role of FoxO1 activation in the cardiac alterations related to cardiac ECM remodeling induced by intrauterine programming in maternal diabetes (Musikant et al., 2019).

CONCLUSION

The mechanisms involved in fetal programming of heart damage in maternal diabetes reviewed in this study include the dysregulation of key players in cardiac cell metabolism, increased damaging effects of a pro-oxidant and proinflammatory environment, and alterations in the cardiac ECM remodeling and intercellular communication. As summarized in **Figure 1**, all these mechanisms and the main players that regulate these altered pathways are interconnected and differentially modulated by epigenetic modifications. This evidences the complex and multiple pathways able to induce programming of cardiovascular diseases in the offspring of diabetic dams, but also, due to the close interaction of these main pathways, brings opportunities to facilitate intervention to provide protective effects. Dietary treatments that improve the maternal metabolism altered by diabetes, such as those with probiotics or supplementation with olive or fish oil, have been shown to have beneficial effects in the cardiac cell metabolism of the offspring. Some of the studies reviewed in this work point to olive oil supplementation in the maternal diet and the maternal treatment with N-acetyl-cysteine as effective treatments able not only to improve cardiac cell metabolism but also to reverse the programmed damaging effect of the pro-oxidant and proinflammatory environment and the alterations in the cardiac ECM remodeling in the offspring heart. Further research addressing plausible treatments in animal models of maternal diabetes and their translation into clinical practice is encouraged.

AUTHOR CONTRIBUTIONS

RH and AJ designed the research. RH and ML conducted the research. RH wrote the manuscript. AJ contributed to the critical reading of the manuscript. All authors contributed to the article and approved the submitted version.

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Caveolin-1/Endothelial Nitric Oxide Synthase Interaction Is Reduced in Arteries From Pregnant Spontaneously Hypertensive Rats

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We have investigated the role caveolae/caveolin-1 (Cav-1) plays in endothelial nitric oxide synthase (eNOS) activation and how it impacts pregnancy-induced decreased vascular reactivity in normotensive (Wistar rats) and spontaneously hypertensive rats (SHR). Wistar rats and SHR were divided into non-pregnant (NP) and pregnant (P). Nitrite levels were assessed by the Griess method in the aorta and mesenteric vascular bed. In functional studies, arteries were incubated with methyl-β-cyclodextrin (dextrin, 10 mmol/L), which disrupts caveolae by depleting cholesterol, and concentration-response curves to phenylephrine (PE) and acetylcholine (ACh) were constructed. Electronic microscopy was used to determine endothelial caveolae density in the aorta and resistance mesenteric artery in the presence of vehicle or dextrin (10 mmol/L). Western blot was performed to evaluate Cav-1, p-Cav-1, calmodulin (CaM), and heat shock protein 90 (Hsp90) expression. Cav-1/eNOS interaction in the aorta and mesenteric vascular bed was assessed by co-immunoprecipitation. Nitric oxide (NO) generation was greater in arteries from P groups compared to NP groups. Dextrin did not change vascular responses in the aorta from P groups or the number of caveolae in P groups compared to NP groups. Compared to NP Wistar rats, NP SHR showed smaller number of caveolae and reduced Cav-1 expression. Pregnancy did not alter Cav-1, CaM, or Hsp90 expression in the aorta or mesenteric vascular bed from Wistar rats or SHR. These results suggest that pregnancy does not alter expression of the main eNOS regulatory proteins, but it decreases Cav-1/eNOS interaction. Reduced Cav-1/eNOS interaction in the aorta and mesenteric vascular bed seems to be an important mechanism to increase eNOS activity and nitric oxide production in pregnant normotensive and hypertensive rats.

Keywords: pregnancy, spontaneously hypertensive rat, aorta - thoracic, mesenteric artery, endothelial nitric oxide synthase, caveolae, caveolin-1

INTRODUCTION

Caveolae are invaginations of the plasma membrane of cells, including endothelial cells, and they consist mainly of cholesterol, sphingolipids, and proteins (Severs, 1981; Yao et al., 2009), such as caveolin (Cav) isoforms. Three types of caveolin have been identified in mammalian cells: Cav-1, Cav-2, and Cav-3 (Okamoto et al., 1998; Shaul and Anderson, 1998). Cav-1 is an essential protein in structural caveolae formation, and it is involved in different signal transduction pathways (Razani and Lisanti, 2001; Patel et al., 2008). In endothelial cells, Cav-1 interacts directly with endothelial nitric oxide synthase (eNOS), keeping eNOS inactive by preventing it from interacting with calmodulin (CaM). Under conditions where specific stimuli increase intracellular calcium, CaM recognizes calcium ions and is activated, binding to and activating eNOS (García-Cardeña et al., 1996; Ju et al., 1997). Due to the Cav-1 inhibitory function on eNOS activity, Cav-1 knockout mice show greater eNOS activity in endothelial cells (Razani et al., 2001). On the other hand, CaM (Forstermann et al., 1991) and heat shock protein 90 (Hsp90) (García-Cardeña et al., 1998) positively regulate eNOS activity.

Hsp90 is a chaperone protein present in eukaryotic cells. Hsp90 association with other proteins regulates cell signaling pathways (Pearl and Prodromou, 2006). Hsp90 recruitment by vascular endothelial growth factor, histamine, and shear stress activates eNOS, increasing nitric oxide (NO) production (García-Cardeña et al., 1998; Russell et al., 2000). eNOS activation by Hsp90 causes endothelium-dependent relaxation of blood vessels (García-Cardeña et al., 1998), whereas inhibition of Hsp90 binding to eNOS reduces NO production (Shah et al., 1999). In addition, Hsp90 appears to increase CaM affinity for eNOS and to promote dissociation between eNOS and Cav-1 (Takahashi and Mendelsohn, 2003; Sessa, 2004).

Pregnancy in normotensive Wistar rats and spontaneously hypertensive rats (SHR) is a physiological process characterized by significant reduction in mean arterial pressure despite increased plasma volume. This phenomenon stems from reduced peripheral vascular resistance associated with decreased pressor responses and reduced contractile response to vasoconstrictors (Barron et al., 1984; Chu and Beilin, 1993a,b; Ballejo et al., 2002; Elias et al., 2008; Stennett et al., 2009; Ognibene et al., 2012;Zancheta et al., 2015; Troiano et al., 2016). Compared to blood vessels from non-pregnant rats, eNOS activity is increased in systemic arteries from late pregnant normotensive and hypertensive rats. Increased eNOS activity, and hence greater NO production, negatively modulates contraction of systemic blood vessels during pregnancy (Zancheta et al., 2015; Troiano et al., 2016). In this study, we hypothesized that pregnancy changes cell signaling involving caveolae, Cav-1, CaM, Hsp90, and eNOS in arteries from normotensive and SHR. No study has analyzed potential alterations in these proteins, caveolae density, or Cav-1/eNOS interaction in arteries from pregnant hypertensive rats. Given that Cav-1, CaM, and Hsp90 control eNOS activity, this study aimed to analyze the role caveolae and Cav-1 play in eNOS modulation and vascular reactivity of arteries from pregnant normotensive and hypertensive rats.

MATERIALS AND METHODS

The Animal Use Ethics Committee of the School of Dentistry of Araçatuba (CEUA-FOA/UNESP) approved all the experiments in this study (protocol n° 2015-00730).

Animals

Female normotensive Wistar rats and SHR (systolic blood pressure – SBP $\geq$ 150 mmHg) aged 12 weeks were divided into non-pregnant (NP Wistar rats and NP SHR) and pregnant (P Wistar rats and P SHR). NP groups were studied in the estrous phase of the estrous cycle, and P groups were studied at the end of pregnancy (18–20th day of pregnancy). For mating, three female rats and one male rat of the same strain (Wistar rats or SHR) were housed in the same box during the night. Day zero of pregnancy was determined by the presence of sperm in the morning vaginal smear. Animals received standard food and water *ad libitum*, and they were kept under controlled temperature (22–24°C) and humidity (45–65%) with light–dark cycles [12-h (hours) light/12-h dark]. SBP was measured by the Tail-Cuff Plethysmography method (PowerLab, ADInstruments, Melbourne, Australia).

Nitrite Level Determination

The Griess reaction, which converts nitrate to nitrite, was used to determine nitrite levels as a NO metabolite (Green et al., 1982) in thoracic aortic and mesenteric vascular bed homogenates. The Griess reagent (50 µl), consisting of sulfanilamide (1%, w/v), naphthylethylenediamine dihydrochloride (0.1%, w/v), and orthophosphoric acid (25%, v/v), was added to the homogenates (50 µl) and incubated at room temperature for 10 min (minutes). Absorbance was measured in a spectrophotometer at 540 nm and compared to known concentrations of a sodium nitrite curve (0–200 µmol/l). Nitrite levels in the samples were normalized to the protein content of the respective thoracic aortic or mesenteric vascular bed samples. To know the magnitude of the increase in nitrite levels in the blood vessels from P Wistar rats and P SHR, the variation (Δ) of nitrite levels was calculated; that is, nitrite levels in P Wistar rats or P SHR were subtracted from nitrite levels in NP Wistar rats or NP SHR, respectively. The results are expressed in µmol/L/µg of protein.

Vascular Reactivity

Thoracic aortic rings (2 mm) were placed between two stainless steel hooks and connected to an isometric force transducer (DMT, ADInstruments, Melbourne, Australia), maintained in a chamber containing Krebs solution (mmol/L): NaCl 130.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 14.9, glucose 5.5, and $CaCl_2$ 1.6; pH 7.4; 95% O_2 and 5% CO_2; 37°C. Rings were stabilized for 30 min at a baseline tension (30 mN), and their vitality was confirmed with high concentration of potassium chloride (KCl, 120 mmol/L). Endothelium integrity was confirmed by acetylcholine-induced relaxation (ACh, 10 µmol/L) in rings pre-contracted with phenylephrine (PE, 1 µmol/L). Rings that showed relaxation

responses to ACh above 70% in SHR and above 90% in Wistar rats were considered to have endothelium. Concentration-response curves to PE and ACh (0.1 nmol/L to 100 μmol/L) were plotted in the presence of vehicle or methyl-β-cyclodextrin (dextrin, 10 mmol/L). Data are expressed as maximum effect (E_{max}) of PE contraction or ACh relaxation, pD_2 (negative logarithm transformation of EC_{50}, the concentration that produced half-maximal contraction or relaxation amplitude), and area under the curve (AUC), in arbitrary units (AUC represents the magnitude of vasoconstriction or vasodilation).

Electron Microscopy

The aorta and resistance mesenteric artery were dissected and incubated with vehicle (control) or dextrin (10 mmol/l) for 60 min, as previously described (Moreira et al., 1996). Arteries rings were fixed by immersion in a solution containing glutaraldehyde (2%) and paraformaldehyde (2%) in sodium cacodylate buffer (0.1 mmol/L) for 24 h. Preparations were kept in sodium cacodylate (0.1 mmol/L), in a freezer, until the next step. Sample infiltration was performed with Araldite resin and pure acetone, combined at different ratios, for 24 h. Next, the material was polymerized with Araldite resin at 60°C for 72 h. Plastic blocks were trimmed, and 0.5 μm semi-thin sections (Leica ultra-microtome, Wetzlar, Germany) were stained with toluidine blue (1%) so that appropriate areas could be selected for ultrathin sectioning (60–70 nm). Ultrathin sections were collected on carbon-coated single-slot grids followed by contrast with uranyl acetate and lead citrate. Electron micrographs were taken at an initial magnification of 10,000×. Micrographs were photographically enlarged to 50,000× on the computer screen so that details of the caveolae could be visualized. Morphometry and quantitative analysis were performed with the ImageJ software (National Institutes of Health, Bethesda, MD, United States). Caveolae were counted in the peripheral cytosolic space next to endothelial cell membranes. The mean of the groups represents the caveolae count in five different images of each rat divided by the number of rats in each group ($n = 4$–5). Results are expressed as the number of caveolae/μm^2 in the endothelial cytoplasm, representing the caveolae density.

Western Blot

The thoracic aorta and mesenteric vascular bed were removed from Wistar rats and SHR, dissected, and stored at −80°C. Tissues were macerated in liquid nitrogen and homogenized in modified RIPA buffer and protease inhibitor with a sonicator (Vibra Cell Sonics, Newtown, United States). Homogenates were centrifuged (10,000 rpm, 4°C, 20 min), and protein concentration in the supernatant was determined by the Lowry method (Lowry et al., 1951). Bovine serum albumin was used as standard. Sixty micrograms (60 μg) of total protein was applied for electrophoresis on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with skimmed milk (5%) at room temperature for 1 h. Membranes were incubated with primary antibodies for anti-Cav-1 (610,406, BD Biosciences, United States) 1:2000, anti-p-Cav-1 (611,338, BD Biosciences, United States) 1:1000, anti-CaM (sc-137,079, Santa Cruz Biotechnology, United States) 1:250, anti-Hsp90 (610,418, BD Biosciences, United States), and anti-β-actin (A5441, Sigma-Aldrich, United States) at 4°C overnight. Then, membranes were incubated with anti-mouse secondary antibodies at room temperature for 1 h. Bands were detected with chemiluminescent substrate for peroxidase and visualized with ImageQuant LAS 500 (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). β-actin was used to normalize results. Band intensity was quantified with the optical densitometry software ImageJ. Results are in arbitrary units.

Co-immunoprecipitation

The co-immunoprecipitation assay was performed according to the manufacturer's protocol (Protein A/G PLUS-Agarose, sc-2003, Santa Cruz, United States) and as already performed by our group (Troiano et al., 2021). Briefly, a pool ($n = 3$) of thoracic aorta and resistance mesenteric bed (three samples per pool) was lysed in octyl-D-glucoside (ODG, 2%) buffer. Supernatant proteins (500 μg) were rotated with anti-eNOS antibody (610297, BD Biosciences, United States) at 4°C overnight, which was followed by rotation with protein A/G PLUS-agarose beads at 4°C for 3 h. Then, samples were washed in Tris-buffer six times. Next, proteins were eluted with Laemmli sample buffer. After that, samples were boiled at 100°C for 10 min, so eNOS and Cav-1 were detected by Western blot. Results are expressed, in arbitrary units, by the ratio between the intensities of Cav-1 expression and immunoprecipitated eNOS expression.

Statistical Analysis

Results are expressed as the mean ± standard error of the mean of obtained values, and *n* indicates the number of rings, arteries, mesenteric vascular bed, or pools used in each group and experiment.

After Shapiro–Wilk normality tests, results were compared between groups by using two-way ANOVA, followed by the *post hoc* Tukey test. To calculate the variation (Δ) of nitrite levels, results were compared between groups by using Student's *t*-test. GraphPad Prism software version 6.0 (GraphPad Software Corporation, La Jolla, CA, United States) was employed. Differences were considered significant when $p < 0.05$.

RESULTS

Pregnancy Increases Nitrite Levels in the Aorta and Mesenteric Vascular Bed From Wistar Rats and SHR

Compared to NP Wistar rats, nitrite levels in aorta and mesenteric vascular bed homogenates from NP SHR were lower (**Figures 1A,B**). Pregnancy increased nitrite levels in the thoracic aorta, and this increase had greater magnitude in Wistar rats

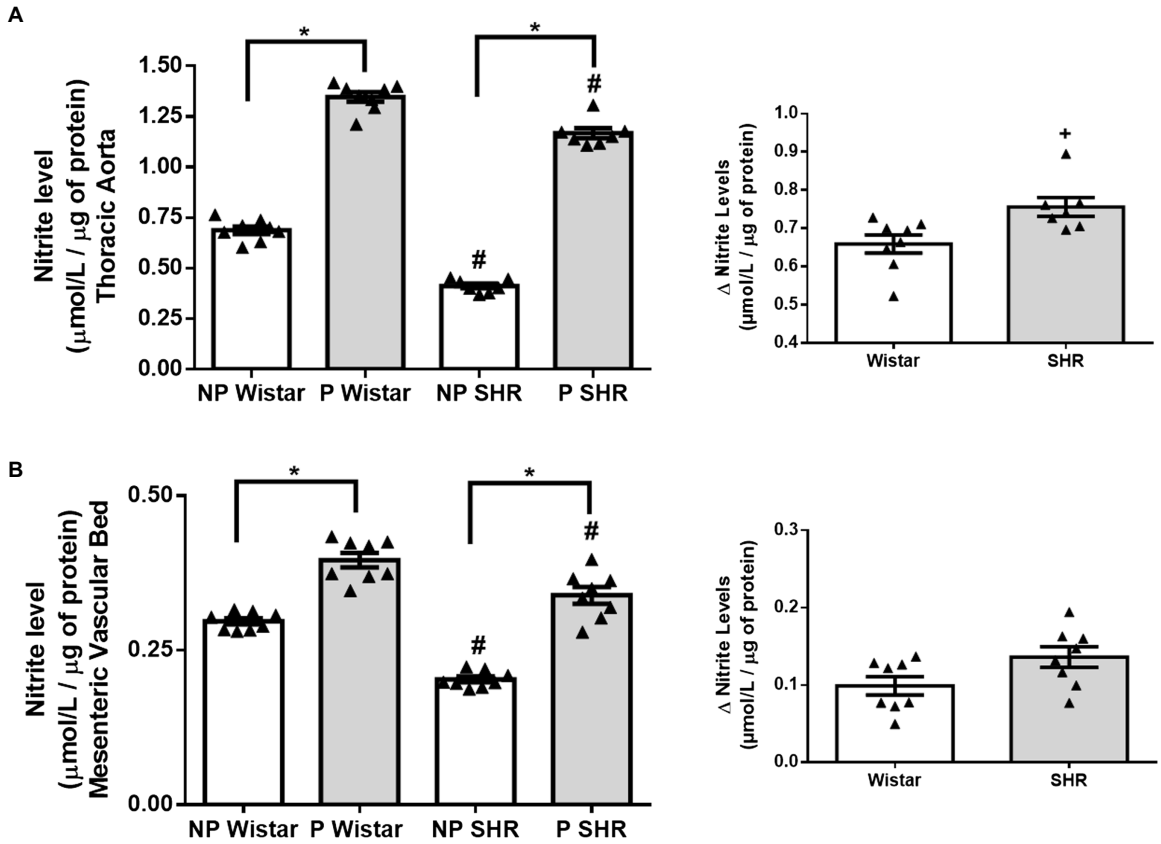

FIGURE 1 | Nitrite levels (µmol/L/ug of protein) and calculation of variation (Δ) of nitrite levels in aortic **(A)** and mesenteric vascular bed **(B)** of non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats. The data represent the mean ± SEM of the results ($n = 7$–8). *$p < 0.05$ P versus NP groups; #$p < 0.05$ NP SHR and P SHR versus NP Wistar rats and P Wistar rats; + $p < 0.05$ SHR versus Wistar rats.

than in SHR. (**Figure 1A**). In mesenteric vascular bed homogenates, pregnancy increased nitrite levels in Wistar rats and SHR similarly (**Figure 1B**, $p = 0.0543$).

Aorta From Pregnant Rats Resists the Effects of Dextrin on PE and ACh Reactivity

We evaluated the effect of dextrin, a cholesterol-depleting agent that disrupts caveolae, on vascular reactivity to PE and ACh and compared these effects for the aorta from NP Wistar rats, P Wistar rats, NP SHR, and P SHR. Dextrin increased PE E_{max} and AUC, in the aorta from NP Wistar rats, but PE potency remained unchanged. Interestingly, dextrin did not alter PE E_{max}, AUC, or potency in P Wistar rats (**Figure 2A**). PE-induced contraction and AUC increased in the presence of dextrin in the aorta from NP SHR, but this drug did not modify PE potency (**Figure 2B**). As in the case of the aorta from P Wistar rats, dextrin did not significantly change PE E_{max}, AUC, or pD_2 in aortic rings from P SHR (**Figure 2B**).

Dextrin did not change ACh E_{max} or potency in the aorta from NP Wistar rats or P Wistar rats compared to the respective controls (**Figure 2C**). The same observation was true for the aorta from NP SHR and P SHR (**Figure 2D**). Although these parameters did not change, dextrin promoted relaxation of smaller magnitude in the aorta from NP Wistar rats and NP SHR compared to the respective NP control curves, while the magnitude of relaxation to ACh remained unaltered in P Wistar rats and P SHR (**Figures 2C,D** – AUC).

Hypertension, Pregnancy, and Dextrin Reduced the Number of Caveolae in the Aorta From Rats

Compared to endothelial cells of the aorta from NP Wistar rats, endothelial cells of the aorta from NP SHR showed reduced number of caveolae (**Figures 3A,B**). The aorta from P Wistar rats presented a smaller amount of endothelial caveolae than the aorta from NP Wistar rats. However, in SHR, pregnancy did not alter the number of caveolae in the aorta.

Treatment of the aorta with dextrin reduced the number of caveolae in NP Wistar rats, but it did not reduce the number of caveolae in the aorta from P Wistar rats (**Figure 3C**). Similarly, dextrin reduced the number of aortic endothelial caveolae in the aorta from NP SHR but not P SHR.

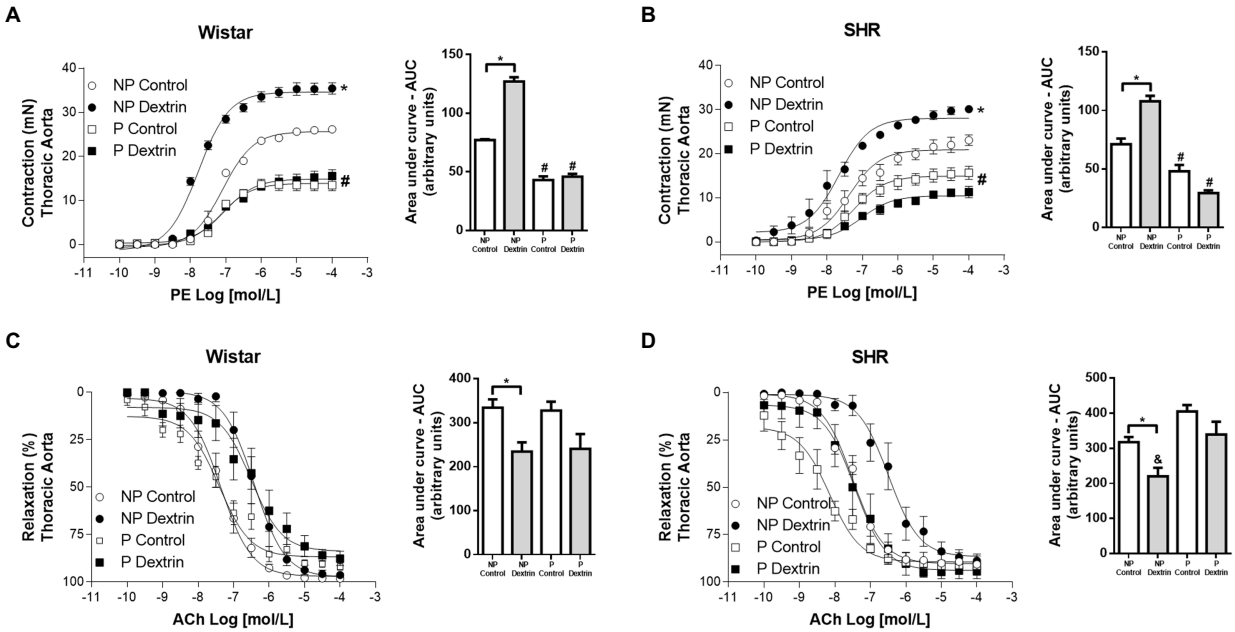

FIGURE 2 | Concentration-effect curves (0.1 nmol/L to 100 μmol/L) to phenylephrine (PE, in **A,C**) and acetylcholine (ACh, in **B,D**) in aortic rings from non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats in the presence of vehicle or dextrin (10 mmol/l). From the concentration-effect curves, we calculated the area under the curve (AUC). The data represent the mean ± SEM of the results ($n = 5$). $^{*}p < 0.05$ E_{max}/AUC NP dextrin versus NP control; $^{\#}p < 0.05$ E_{max}/AUC P control and P dextrin versus NP control and NP dextrin; $^{\&}$AUC NP dextrin versus P control and P dextrin.

Hypertension and Dextrin, but Not Pregnancy, Reduced the Number of Caveolae in the Resistance Mesenteric Artery From Rats

Compared to the resistance mesenteric artery from NP Wistar rats and P Wistar rats, the resistance mesenteric artery from NP SHR and P SHR presented a reduced number of caveolae (**Figures 4A,B**). The number of endothelial caveolae in the resistance mesenteric artery from P Wistar rats was not statistically different from the number of endothelial caveolae in the resistance mesenteric artery from NP Wistar rats. In addition, there were no differences between the number of endothelial caveolae in the resistance mesenteric artery from NP SHR and P SHR (**Figures 4A,B**).

Dextrin reduced the number of caveolae in the resistance mesenteric artery from NP Wistar rats and NP SHR. However, dextrin did not alter the number of caveolae in the mesenteric artery from P Wistar rats or P SHR.

Hypertension, but Not Pregnancy, Reduced Cav-1 and Phosphorylated Cav-1^{Tyr14} Expression in the Aorta From Rats

Compared to NP Wistar rats, Cav-1 expression was lower in aortic homogenates from NP SHR (**Figure 5A**). Still compared to NP Wistar rats, Cav-1 expression did not change in aortic homogenates from P Wistar rats (**Figure 5A**). Aortic homogenates from P SHR and NP SHR did not have statistically different Cav-1 expression (**Figure 5A**).

Compared to NP Wistar rats, the phosphorylated Cav-1^{Tyr14}/total Cav-1 ratio was reduced in aortic homogenates from NP SHR. Pregnancy did not modify p-Cav-1^{Tyr14} expression in aortic homogenates from Wistar rats or SHR (**Figure 5B**). The mesenteric bed homogenates from the groups did not differ in terms of Cav-1 expression (**Figure 5C**) or p-Cav-1^{Tyr14} expression (**Figure 5D**).

Reduced CaM Expression Was Observed in the Aorta, but Not in the Mesenteric Bed From SHR and Pregnancy Did Not Alter CaM Expression

Compared to NP Wistar rats, CaM expression was decreased in aortic homogenates from NP SHR (**Figure 6A**). Pregnancy did not change CaM expression in aortic homogenates from Wistar rats or SHR (**Figure 6A**). CaM expression in mesenteric bed homogenates was not different in Wistar rats and SHR, and pregnancy did not change expression of this target protein (**Figure 6B**).

Neither Hypertension nor Pregnancy Altered Hsp90 Expression in the Aorta or Mesenteric Bed From Wistar Rats or SHR

Compared to NP Wistar rats, Hsp90 protein expression was not altered in blood vessels from NP SHR (**Figures 6C,D**). Hsp90 expression was similar in aortic homogenates from NP Wistar rats and P Wistar rats (**Figure 6C**). Pregnancy did not modify Hsp90 expression in mesenteric bed homogenates (**Figure 6D**) from Wistar rats and SHR.

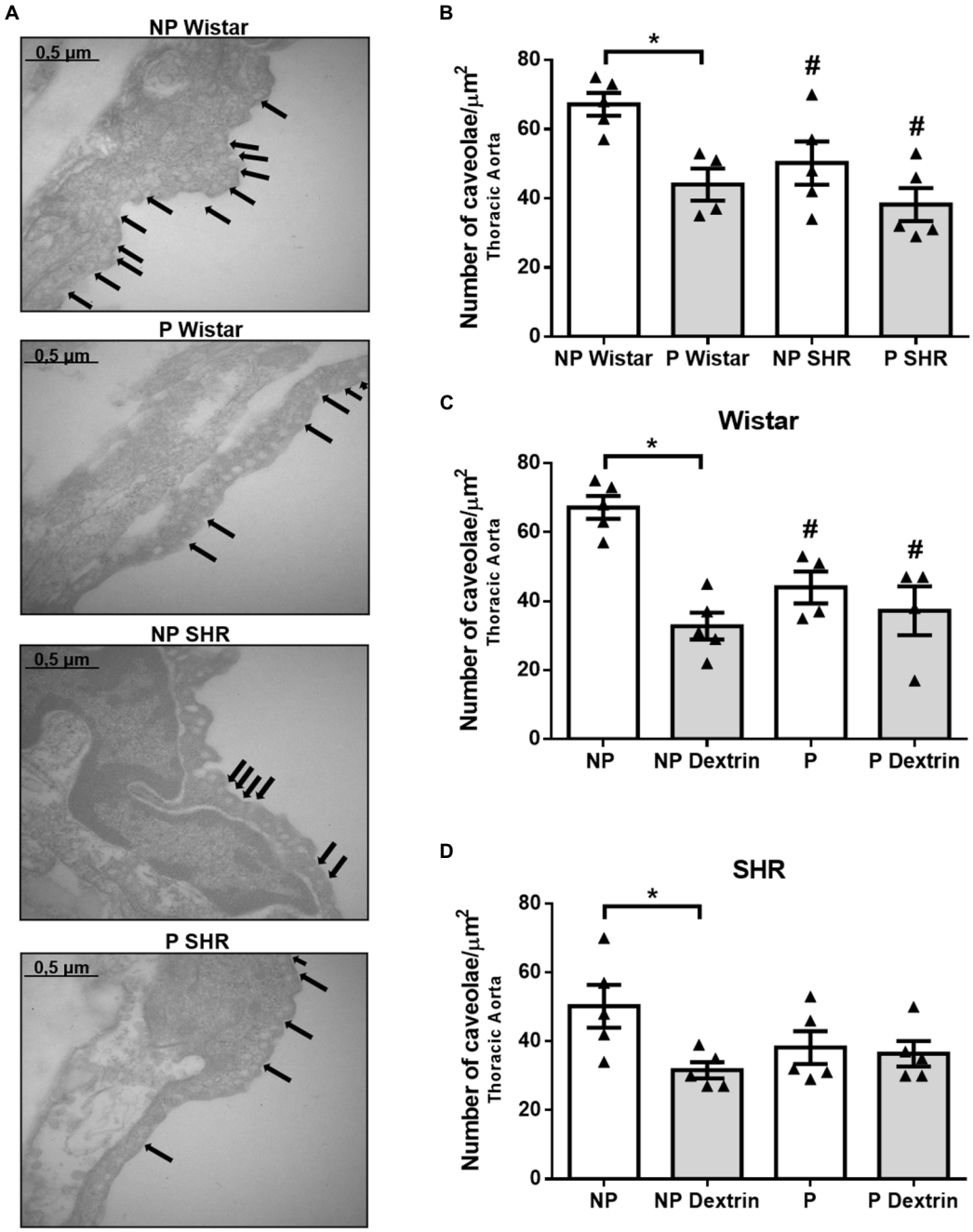

FIGURE 3 | Representative photomicrographs (50,000× magnification, in **(A)** and quantification of the number of endothelial caveolae/µm^2 in aorta of non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats **(B)** in the presence of vehicle or dextrin (10 mmol/L, **C,D**). The bars represent the mean ± SEM of the results obtained in the aorta (n = 4–5) from the different groups. *p < 0.05 P Wistar rats or P dextrin versus NP groups; # NP SHR or P SHR versus NP Wistar rats.

Cav-1/eNOS Interaction Is Reduced by Hypertension and Pregnancy in Female Rat Blood Vessels

Compared to NP Wistar rats, Cav-1/eNOS interaction in aortic and mesenteric vascular bed homogenates decreased in NP SHR (**Figures 7A,B**). Pregnancy reduced Cav-1/eNOS interaction in the aorta (**Figure 7A**) and mesenteric vascular bed (**Figure 7B**) from Wistar rats and SHR.

DISCUSSION

Caveolae are an important structure in vascular cells AND most eNOS is in these invaginations of the plasma membrane. Within the plasma membrane, eNOS is highly expressed in caveolae, and its activity is seven times greater in the plasma membrane than in the cytosol. eNOS activity is not detected in membrane fractions without caveolae. Furthermore, in

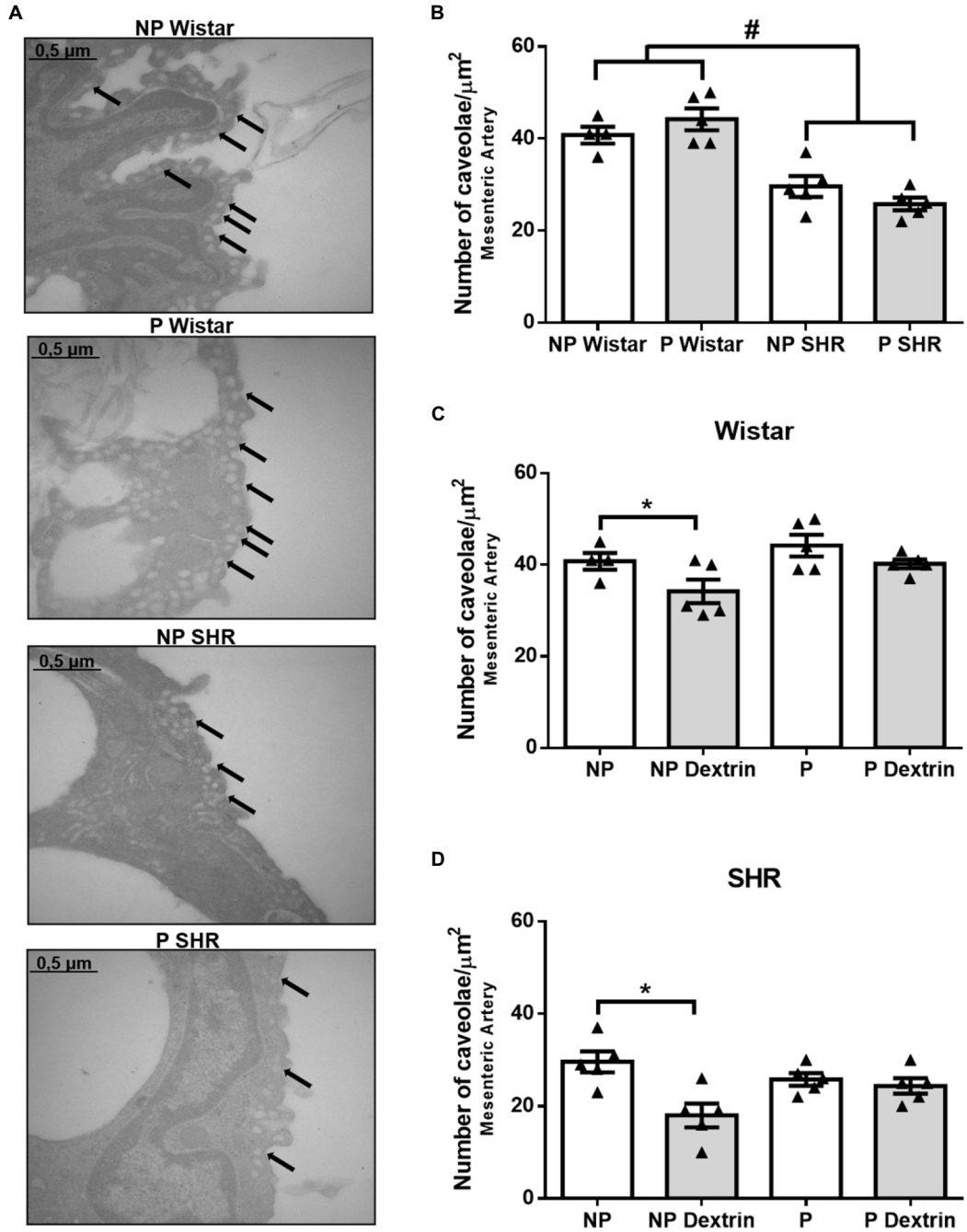

FIGURE 4 | Representative photomicrographs (50,000× magnification, in **(A)** and quantification of the number of endothelial caveolae/µm^2 in resistance mesenteric arteries of non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats **(B)** in the presence of vehicle or dextrin (10 mmol/L, **C,D**). The bars represent the mean ± SEM of the results obtained in the resistance mesenteric artery ($n = 5$) from the different groups. $^{\#}p < 0.05$ NP SHR and P SHR versus NP Wistar rats and P Wistar rats; * < 0.05 NP dextrin versus NP.

resting conditions, most functional eNOS is in the caveolae, where between 57 and 100% eNOS activity is detected (Shaul et al., 1996). Accordingly, caveolae contain specific proteins that participate in eNOS signaling (Li et al., 1995; Chini and Parenti, 2004; Wang et al., 2005; Bernatchez et al., 2011).

We observed greater amount of NO in the aorta and mesenteric bed at the end of pregnancy, as indirectly measured by nitrite levels (**Figures 1A,B**). This increase in NO may be associated with increased total eNOS and p-eNOS^{Ser1177} expression in the aorta and higher NO concentration in aortic endothelial cells from P Wistar rats and P SHR, as already

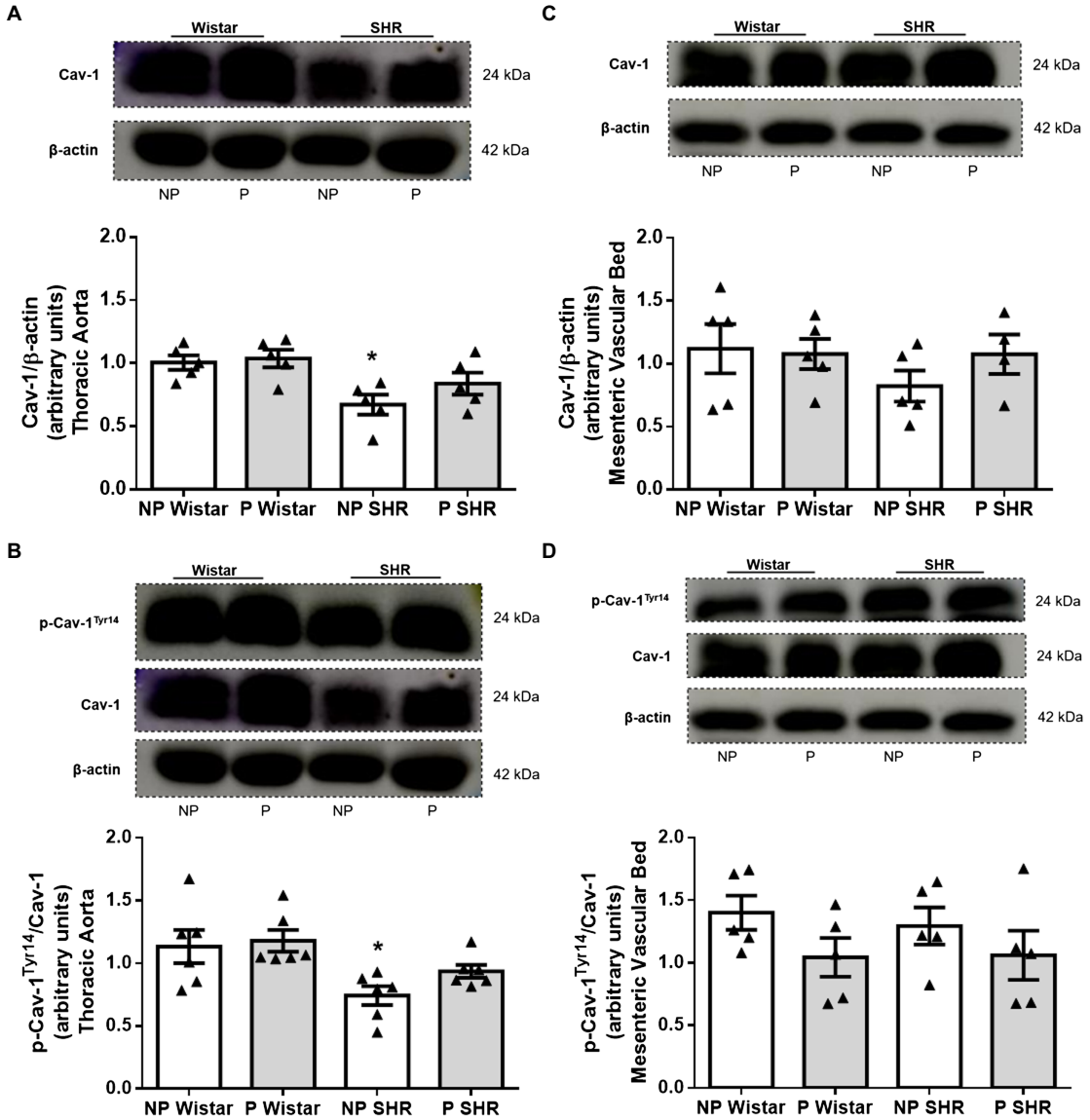

FIGURE 5 | Representative images and quantification of the caveolin-1 (Cav-1)/β-actin **(A,C)** and p-Cav-1^{Tyr14}/Cav-1 **(B,D)** expression in the aorta and resistance mesenteric artery homogenates of non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats. The bars represent the mean ± SEM of the results obtained in the aorta ($n = 5$) from the different groups. *$p < 0.05$ NP SHR versus NP Wistar rats and P Wistar rats.

observed by our research team (Zancheta et al., 2015; Troiano et al., 2016). Moreover, in the aorta, where NO is the main regulatory factor of vascular homeostasis, variation of NO levels is greater in NP SHR and P SHR than in NP Wistar rats and P Wistar rats, emphasizing the importance of this molecule for regulation of vascular mechanisms during pregnancy of hypertensive rats.

To evaluate the role caveolae play in PE-induced contraction and ACh-induced relaxation, we performed functional reactivity studies on the aorta from NP Wistar rats, P Wistar rats, NP SHR, and P SHR in the presence of dextrin, a drug that disrupts the caveolae structure (Linder et al., 2005). As expected, dextrin disrupts the caveolae structure, compromising the eNOS function and favoring contractile effects, thereby increasing PE reactivity in the aorta from NP Wistar rats and NP SHR (**Figures 2A,B**). Dextrin increases PE-stimulated vascular contraction by a mechanism associated with lower eNOS-derived NO production in aortic rings (Potje et al., 2019) and femoral arteries from Wistar rats (Al-Brakati et al., 2015). In our study, dextrin shifted the concentration-responses curves to ACh to the right, and a lower magnitude of relaxation is observed only in vessels from NP Wistar rats and NP SHR (**Figures 2C,D**). These data reinforce the importance of stable caveolae for eNOS localization and activity in blood vessels from normotensive and hypertensive rats. Corroborating our data, dextrin impairs ACh-induced vasodilation in male normotensive rats, such as

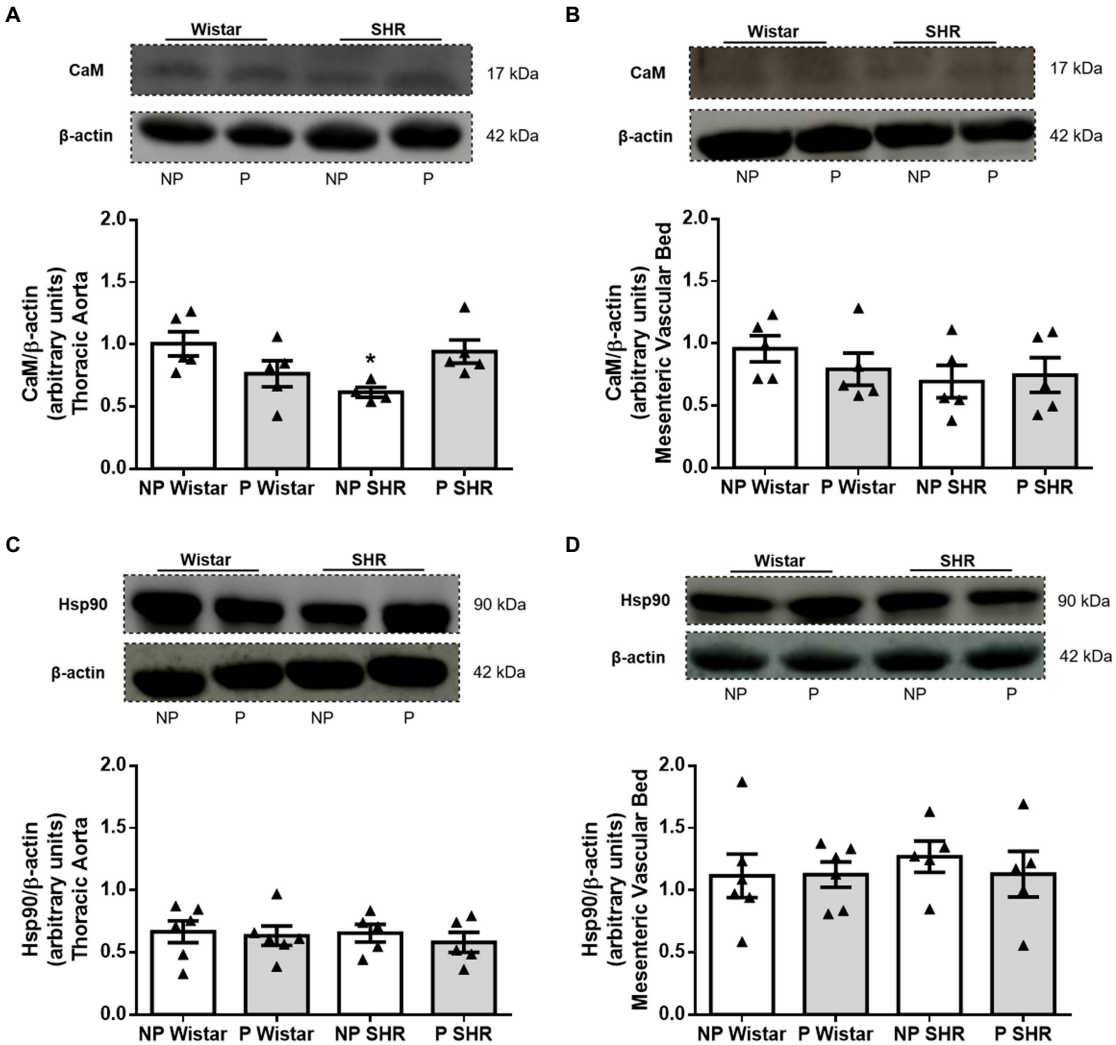

FIGURE 6 | Representative images and quantification of calmodulin (CaM)/β-actin and heat shock protein 90 (Hsp90)/β-actin expression in the aorta (in **A,C**) and resistance mesenteric bed (in **B,D**) homogenates from non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats. The bars represent the mean ± SEM of the results obtained in the aorta and resistance mesenteric bed ($n = 5–6$) from the different groups. *$p < 0.05$ NP SHR versus NP Wistar rats.

Sprague–Dawley (Linder et al., 2005) and Wistar (Rodrigues et al., 2009; Potje et al., 2019), and also in male hypertensive rats models, such as SHR (Potje et al., 2019) and two kidneys, one clip (2K-1C) (Rodrigues et al., 2009).

Interestingly, dextrin has no effects on PE contraction or ACh relaxation in the aorta from P Wistar rats or P SHR, suggesting that eNOS activity is less dependent on caveolae stability during pregnancy. Rizzo and collaborators (Rizzo et al., 1998) showed that increased vascular flow and pressure generates hemodynamic forces *in situ* that activate caveolar eNOS, thus dissociating eNOS from caveolin and favoring its association with CaM at the luminal endothelial cell surface, but not in caveolae plasma membrane. Additionally, after prolonged agonist stimulation, eNOS undergoes depalmitoylation and translocates to specific interior cell compartments or the cytosol (Michel et al., 1997; Yeh et al., 1999). These studies demonstrated that active eNOS is not present in caveolae structure, reinforcing our hypothesis that eNOS function is less dependent on caveolae stability during pregnancy. Besides that, higher NO production observed in arteries from pregnant rats (**Figure 1**) confirms increased eNOS activity during pregnancy.

We further investigated whether pregnancy alters the number of caveolae present in the endothelium of the aorta and resistance mesenteric artery from Wistar rats and SHR. By using electron microscopy, we observed that, compared to P-Wistar rats, the number of endothelial caveolae in the aorta and mesenteric artery from P SHR is reduced (**Figures 3B, 4B**). These results corroborate previous findings by our group of reduced number of caveolae in the aorta and mesenteric artery of male SHR compared to matched arteries in male Wistar rats (Potje et al., 2019). Compared to normotensive rats, male hypertensive 2K-1C rats also exhibit reduced number of aortic endothelial and

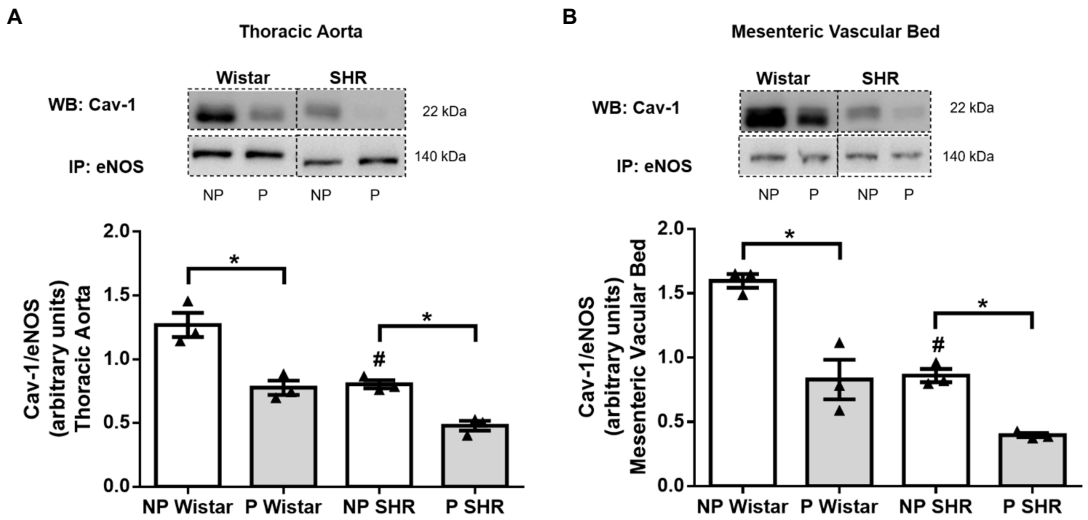

FIGURE 7 | Analysis of Cav-1/eNOS interaction by co-immunoprecipitation (Co-IP) of endothelial nitric oxidase synthase (eNOS) associated with caveolin-1 (Cav-1) immunoblot (WB) in the aorta **(A)** and resistance mesenteric bed **(B)** of non-pregnant (NP) and pregnant (P) Wistar and spontaneously hypertensive (SHR) rats. The bars represent the mean ± SEM of the results obtained in pools of the aorta and mesenteric bed ($n = 3$) from the different groups. $^{*}p < 0.05$ P versus NP groups; $^{\#}p < 0.05$ NP SHR versus NP Wistar rats.

smooth cell caveolae (Rodrigues et al., 2007; Rodrigues et al., 2009). Together, these results show that hypertension is associated with reduced amount of caveolae in arteries, which contributes to vascular dysfunction.

In Wistar rats, pregnancy significantly reduces the number of aortic endothelial caveolae (**Figure 3B**), but this effect seems to depend on the arterial bed because we did not observe it in the mesenteric artery (**Figure 4B**). Pregnancy does not change the caveolae density in the aorta or resistance mesenteric artery of P SHR (**Figures 3B, 4B**) compared to NP SHR. As expected, dextrin reduces the number of caveolae only in arteries from NP Wistar rats. However, in arteries from P Wistar rats and P SHR, dextrin does not modify the number of caveolae (**Figures 3C,D** and **4C,D**). These results reinforce the suggestion that endothelial caveolae in pregnant rat arteries are more resistant to the effects of dextrin. A higher dextrin concentration may be needed to produce the same effect observed in vessels from non-pregnant rats.

We evaluated possible changes in the expression of proteins that control eNOS activity in pregnant rat arteries. Compared to Wistar rats, Cav-1 expression is reduced in the aorta, but not in the mesenteric artery from SHR. This result reinforces data showing decreased Cav-1 expression in male SHR aorta (Piech et al., 2003; Sánchez et al., 2006; Vera et al., 2007; Cristofaro et al., 2012), which may account for reduction in the number of caveolae. Pregnancy does not alter Cav-1 expression in vessels from Wistar rats or SHR (**Figures 5A,C**), but reduced the caveolae density only in the aorta from Wistar rats. Therefore, changes in caveolae density are not essentially followed by alterations in Cav-1 expression as reported by our group (Potje et al., 2019). Phosphorylated Cav-1^{Tyr14} binds to eNOS and consequently inactivates the enzyme. However, this mechanism compensates for the increase in $eNOS^{Ser1177}$ phosphorylation *via* NO/Src, that is, increased NO due to increased $eNOS^{Ser1177}$ phosphorylation would activate p-Src, causing Cav-1^{Tyr14} phosphorylation, which in turn increases eNOS binding/inhibition (Chen et al., 2012). Compared to NP Wistar rats and P Wistar rats, Cav-1^{Tyr14} phosphorylation is reduced in the aorta from NP SHR, which may suggest that lower Cav-1 activity stems from lower NO production (**Figure 5B**).

Compared to NP Wistar rats, aortic CaM expression is reduced in NP SHR, corroborating previous results showing reduced CaM expression in male SHR compared to normotensive rats (Piech et al., 2003; Sánchez et al., 2006; Vera et al., 2007; Cristofaro et al., 2012). Pregnancy does not alter CaM expression in arteries from Wistar rats or SHR (**Figures 5A,C**). Different studies have shown that ACh-induced relaxation is not altered at the end of pregnancy in Wistar rats (Ballejo et al., 2002; Zancheta et al., 2015). These data suggest that pregnancy does not play a role in direct activation of the Ca^{2+}-Calmodulin complex or changes in CaM expression.

Hsp90 binding to eNOS stimulates eNOS activity, increasing the catalytic function of the enzyme and maintaining balance between eNOS-derived NO and superoxide anion (Brouet et al., 2001; Pritchard et al., 2001). Experiments on human umbilical vein endothelial cells and isolated rat aorta have shown that eNOS activity increases when a complex between eNOS and Hsp90 is formed, increasing NO production (Wang et al., 2009). The link between Hsp90 expression and phosphorylated eNOS in male SHR aortic tissues is lower compared to normotensive rats (WKY), and this relationship increases when SHR are treated with sodium nitrite (Ling et al., 2016). To date, our results have shown that total Hsp90 expression remains unchanged in arteries from NP SHR arteries compared to arteries from NP Wistar rats (**Figures 5B,D**), suggesting that Hsp90 appears not to be involved in eNOS activation in arteries from pregnant rats. However, it has been demonstrated that Hsp90 subunits play a specific role

in eNOS activity, with Hsp90α causing Akt and $eNOS^{Ser1177}$ phosphorylation, increasing NO production; and with Hsp90β leading to $eNOS^{Thr495}$ phosphorylation, producing superoxide anion (Tanaka et al., 2015). Further studies should be carried out to evaluate possible changes in Hsp90α expression and activity in arteries from pregnant rats.

We also analyzed Cav-1/eNOS interaction. As reported throughout this manuscript, decreased eNOS/Cav-1 interaction increases eNOS activity and NO production. eNOS/Cav-1 interaction is reduced in arteries from SHR compared to Wistar rats, which is followed by smaller amounts of NO in hypertensive vessels. Impaired NO production and signaling in hypertension has been attributed to eNOS uncoupling, which is associated with tetrahydrobiopterin (BH4) oxidation, L-arginine deficiency, eNOS S-glutathionylation, eNOS-dependent superoxide production, or increased NOX activity (Li et al., 2015). Therefore, less interaction in SHR is not associated with higher NO levels because eNOS may be uncoupled during hypertension. In addition, pregnancy further decreases eNOS/Cav-1 interaction in arteries from Wistar rats and SHR (**Figures 7A,B**), which may contribute to higher NO levels (**Figure 1**) and greater eNOS activity, as already shown (Zancheta et al., 2015). eNOS may translocates to other cell compartments, with its specific localization in caveolae not being mandatory at the end of pregnancy.

Taken together, our data demonstrated that eNOS function is less dependent on caveolae stability during pregnancy and that pregnancy does not alter the main eNOS regulatory proteins expression, but it decreases Cav-1/eNOS interaction. In conclusion, reduced Cav-1/eNOS interaction in the aorta and mesenteric vascular bed seems to be an important mechanism to increase eNOS activity and nitric oxide production in blood vessel from pregnant normotensive and hypertensive rats.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Use Ethics Committee of the School of Dentistry, São Paulo State University (UNESP), Aracatuba, São Paulo, Brazil.

AUTHOR CONTRIBUTIONS

JT, SP, and CA conceived and designed the experiments. JT, SP, MG, and EG performed the experiments. JT, SP, RT, and CA wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.760237/full#supplementary-material

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frontiers in Physiology

REVIEW
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Supraphysiological Role of Melatonin Over Vascular Dysfunction of Pregnancy, a New Therapeutic Agent?

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Hypertension can be induced by the disruption of factors in blood pressure regulation. This includes several systems such as Neurohumoral, Renin-angiotensin-aldosterone, the Circadian clock, and melatonin production, which can induce elevation and non-dipping blood pressure. Melatonin has a supraphysiological role as a chronobiotic agent and modulates vascular system processes via pro/antiangiogenic factors, inflammation, the immune system, and oxidative stress regulation. An elevation of melatonin production is observed during pregnancy, modulating the placenta and fetus's physiological functions. Their impairment production can induce temporal desynchronization of cell proliferation, differentiation, or invasion from trophoblast cells results in vascular insufficiencies, elevating the risk of poor fetal/placental development. Several genes are associated with vascular disease and hypertension during pregnancy via impaired inflammatory response, hypoxia, and oxidative stress, such as cytokines/chemokines IL-1β, IL-6, IL-8, and impairment expression in endothelial cells/VSMCs of HIF1α and eNOS genes. Pathological placentas showed differentially expressed genes (DEG), including vascular genes as CITED2, VEGF, PL-II, PIGF, sFLT-1, and sENG, oncogene JUNB, scaffolding protein CUL7, GPER1, and the pathways of SIRT/AMPK and MAPK/ERK. Additionally, we observed modification of subunits of NADPH oxidase and extracellular matrix elements, i.e., Glypican and Heparanase and KCa channel. Mothers with a low level of melatonin showed low production of proangiogenic factor VEGF, increasing the risk of preeclampsia, premature birth, and abortion. In contrast, melatonin supplementation can reduce systolic pressure, prevent oxidative stress, induce the activation of the antioxidants system, and lessen proteinuria and serum level of sFlt-1. Moreover, melatonin can repair the endothelial damage from preeclampsia at the placenta level, increasing PlGF, Nrf-2, HO-1 production and reducing critical markers of vascular injury during the pregnancy. Melatonin also restores the umbilical and uterine blood flow after oxidative stress and inhibits vascular inflammation and VCAM-1, Activin-A, and sEng production. The beneficial effects of melatonin over pathological pregnancies can be partially observed in normal pregnancies, suggesting the dual role of/over placental physiology could contribute to protection and have therapeutic applications in vascular pathologies of pregnancies in the future.

Keywords: melatonin, hypertension, pregnancy, vascular, preeclampsia

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INTRODUCTION

The control of blood pressure results from the contribution of several tissues and neural circuits via the multifactorial interaction of several physiological factors such as the heart rate, cardiac output, and peripheral resistance. The peripheral resistance determines the peripheral blood circulation, dependent on the arterial and venous tone. The chronic elevation of blood pressure (persistently raised pressure, >140 mmHg/90 mmHg) or hypertension is a severe medical condition associated with elevated risk factors for morbidity and mortality worldwide, a major cardiovascular risk factor. These pathologies are present between 20 and 25% of the population, or about 1.13 billion people worldwide (World Health Organization, and World Health Organization, 2013), and affect about 8% of reproductive-aged women, representing about 688 million women (Mupfasoni et al., 2018; Braunthal and Brateanu, 2019). The severe expression of hypertension is named malignant-hypertension or accelerated-hypertension, affecting about 2-7 cases per 100,000 habitants. This rate increases every year (Shantsila and Lip, 2017), suggesting that this pathology and its more extreme variations have become a massive health problem in terms of morbidity and mortality worldwide.

Several factors modulate vascular circulation and blood pressure, which can be divided into intrinsic and extrinsic pathways, modulating the vascular tone, coagulation, and the vascular system's flow. Intrinsic regulation pathways involve the paracrine production of endothelial cells, periadventitial adipose tissue, and vascular smooth muscle cell. The extrinsic regulation factor involves neuronal regulation such as sympathetic/parasympathetic innervation and the humoral secretion from the endocrine system. The intrinsic occurs via the paracrine liberation of cytokines, gasotransmitters, growth factors, vasoactive peptides, vascular protective agents, anticoagulant, angiogenic peptides, and others, which maintain the vasomotor and mitogenic balance required for an adequate vascular tone in the peripheral circulation (Konukoglu and Uzun, 2017; Gheibi et al., 2018; Oparil et al., 2018). These complex interactions require a supraphysiological regulation that includes the participation of the neurohumoral system that includes the renin-angiotensin-aldosterone system (RAAS), the circadian system, and melatonin production by the pineal gland see **Figure 1** (Baker and Kimpinski, 2018; Nakashima et al., 2018; Oparil et al., 2018; Zuo and Jiang, 2020). Disruption of the intrinsic or extrinsic factors involved in blood pressure regulation can induce elevation and non-dipping blood pressure resulting in damage over vascular cells or tissues. Moreover, these factors can be affected by nutrition, environment, fetal programming, adiposity, diet, sodium and potassium intake, alcohol intake, smoking, physical activity, air pollution, and stress which give multivariable causes and expressions for hypertension (NCD Risk Factor Collaboration (Ncd-RisC), 2017). However, multifactorial gene-environment etiology is associated with 90–95% of patients with primary hypertension, besides showing an association with a genetic component in about 35-50% of patients, suggesting the relevance of finding new pathways and new molecular markers to help predict the risk of morbidity and mortality by hypertension (Oparil et al., 2018).

Melatonin has a role as a chronobiotic agent synchronizing the circadian system and plays a supraphysiological role in modulating other vascular system processes via modulation of inflammation, the immune system, and oxidative stress. This role was first described in the late-1960s in Pinealectomy in rats (Zanoboni and Zanoboni-Muciaccia, 1967). This study observed an increase of 30% in blood pressure (hypertension) at 15 days after surgery (Zanoboni and Zanoboni-Muciaccia, 1967). Melatonin supplementation can partially revert the harmful effects of this hypertension, as lipoperoxidation, hydroxyl radical generation, superoxide anion radical, and inducing antioxidant capacity via increased glutathione (GSH) content (Mukherjee et al., 2010). Moreover, melatonin plays a protective role in restoring hemodynamic parameters after myocardial injury, explaining blood pressure reduction in pathological conditions (Mukherjee et al., 2010), induced arterial vasorelaxation after vasoconstrictor treatment, and prevented the vasoconstriction at the level of cerebral arteries (Torres-Farfan et al., 2008; Qiu et al., 2018). Patients treated with melatonin supplementation reduce by about 10% in the MAP and SBP, not altering the heart rate (Bazyar et al., 2021), suggesting a protective role over the cardiovascular function of melatonin hormone by their capacity antioxidant and hypotensive role over the cardiovascular system.

The cardiovascular disease of the mother/offspring induces an adverse intrauterine environment which gives outputs such as fetal hypoxia, intrauterine growth restriction, gestational hypertension, and Preeclampsia. During normal pregnancy, melatonin production increase depending on gestational age and falls immediately after delivery (Nakamura et al., 2001; Ejaz et al., 2020). The impaired melatonin production is associated with complications during pregnancy, such as severe Preeclampsia, hypertension, and proteinuria. During severe Preeclampsia, Melatonin levels in women are reduced (Dou et al., 2019). However, melatonin supplementation can reduce oxidative stress and hypertension during pregnancy. Suggesting that melatonin production during pregnancy maintains cardiovascular health, reducing premature birth and abortion (Valenzuela et al., 2015; de Chuffa et al., 2019).

However, potentially, another genetic component can work during hypertension, and these genes can be modulated by melatonin. For this purpose, we searched the Differentially Expressed Genes (DEG) during hypertension detected in the tone regulation by vascular smooth muscle contraction. We found 36 upregulated and downregulated genes in vascular smooth muscle contraction pathways during hypertension see **Table 1**. This suggests that there are a number of pathways involved in this pathology and the complex pathways involved in vascular smooth muscle.

HYPERTENSION AND PREGNANCY

The American College of Obstetricians and Gynecologists (ACOG) defines hypertension during pregnancy as a pressure ≥ 140/90 mm Hg for systolic and/or diastolic BP

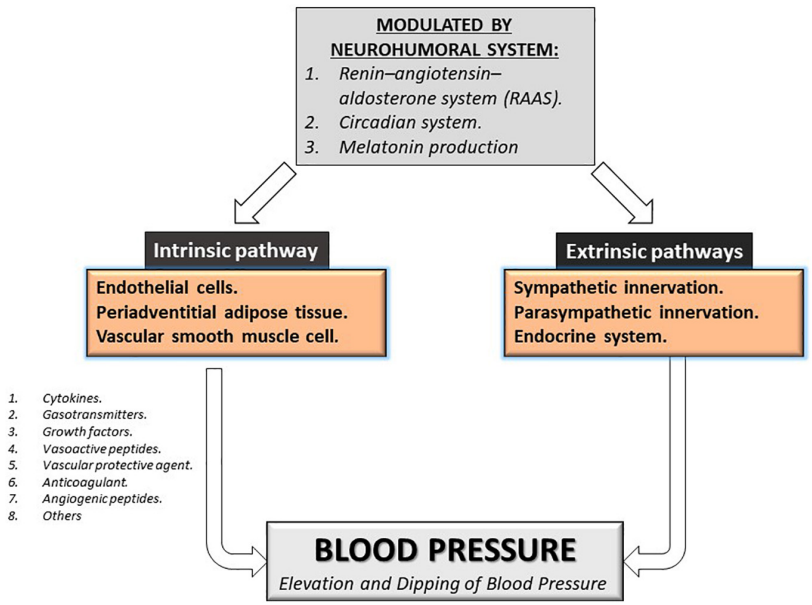

FIGURE 1 | Modulation of blood pressure. Intrinsic and extrinsic pathways can modulate blood pressure. The principal regulator of both pathways is the neurohumoral system Renin-Angiotensin-aldosterone system, the circadian system, and melatonin production.

(Bello et al., 2021). Following this criterion, hypertension can affect about 8% of women of reproductive age and present in about 10% of pregnancies (Braunthal and Brateanu, 2019).

TABLE 1 | Genes into vascular smooth muscle altered by hypertension that could alter contraction pathways.

Differentially expressed genes associated with Hypertension *(N = 36 genes)*			
ARAF	*GUCY1A3*	*NPR2*	*PRKCG*
GNAS	*GUCY1B3*	*PLA2G2A*	*PPP1CC*
RAF1	*ITPR1*	*PLA2G4A*	*RHOA*
ARHGEF11	*ITPR2*	*PLA2G6*	*RAMP1*
ADCY2	*ITPR3*	*PLA2G2C*	*RAMP2*
ADCY5	*MAPK1*	*PLA2G5*	*RAMP3*
ADCY6	*MAP2K1*	*PLCB4*	
ADRA1D	*MAP2K2*	*KCNMB4*	
AGTR1A	*MYLK2*	*PRKCD*	
CALM3	*NPR1*	*PRKCH*	

Analyses of datasets from the GEO database (http://www.ncbi.nlm.Nih.gov/geo) available for hypertension were performed in the GEO database (n = 73). We excluded the platforms without gene accession numbers, incomplete incoming information, and results of peripheral blood. Platforms GSE74288, GSE113613, GSE89073, GSE105114, GSE105114, GSE105114, GSE84704, GSE53363, GSE72707, GSE72181, GSE64613, GSE69601, GSE46863, GSE53408, GSE45927, GSE59437-BRAIN, GSE50833, GSE48936, GSE43292, GSE40182, GSE26671, GSE30428, GSE24988, GSE19817, GSE16624, and GSE5488 were visualized using the GEO Profile graphics a web tool to compare the two groups using Benjamin and Hochberg false discovery rate methodology, using the parameter by default (logFC $\geq$ 1 and adj. $P < 0.05$). For the functional analysis, we used the Kyoto Encyclopedia and Genes and Genomes (KEGG) and selected vascular smooth muscle contraction pathways. The gene expression profile was combined and identified with Venn Diagram, we then undertook the identification of DEGs.

During the pregnancy, about 4,3% corresponded to chronic hypertension, and 6% were defined as gestational hypertension, which during the pregnancy elevates the negative outputs for the mother and fetus, such as preeclampsia, preterm birth, and the baby being small for gestational age (Bello et al., 2021). Impaired placentation is the principal cause of complications in pregnancy. It causes several negative outputs, including hypertension, and their severe expression occurs in about 2% and induces about 16% of all maternal deaths suggesting the relevance of prevention of hypertension and preeclampsia (Romero et al., 2011; Poniedziałek-Czajkowska et al., 2021).

Preeclampsia involves the vasculature due to the impaired transformation of the spiral arteries and the reduced perfusion to the fetus and the placenta, with an elevation of oxidative and endoplasmic reticulum stress and finally, inducing a fetal growth restriction. The systemic stress shoddy remodeling of the uteroplacental spiral arteries release placental factors to maternal circulation, increasing the maternal inflammatory response and oxidative stress such as high production of proinflammatory cytokines/chemokines such as IL-1β, IL-6, and IL-8 (Nunes et al., 2019; Valencia-Ortega et al., 2019; Poniedziałek-Czajkowska et al., 2021; Spence et al., 2021). Moreover, the impaired invasion of the uterine wall by trophoblast induce hypoxia, and produce a modification of placental secretion of critical angiogenic and antiangiogenic factors such as vascular endothelial growth factor (VEGF) (Frigato et al., 2009) and Placental lactogen (PL-II) member of the prolactin gene family. These vascular factors and their impaired secretion have been proposed to predict risk during pregnancy (Wang and Zhao, 2010; Valenzuela et al., 2012; Lenke et al., 2019). Besides, VEGF and placental growth factor (PlGF) induce placental angiogenesis via activation of VEGFR-1/Flt-1 and VEGFR-2/KDR, and both factors increase

endothelial cell adhesion, chemotaxis and increase angiogenesis (Helske et al., 2001; Poniedziałek-Czajkowska et al., 2021).

Several antiangiogenic factors are highly secreted during pathological pregnancies, such as Fms-like tyrosine kinase-1 (sFlt-1), which is the soluble secretion of VEGF Receptor-1, and their secretion by villous cytotrophoblasts cells induced by hypoxia during Preeclampsia via activation of HIF1α. Another antiangiogenic factor secreted during Preeclampsia is soluble Endoglin (sEng), which reduces the proangiogenic and vasodilator effects of Endoglin. Finally, the elevated production of sFlt-1 and sEng and the decrease of VEGF and PIGF secretion induce an impairment of endothelial function, causing preeclampsia (De Oliveira et al., 2013; Shah and Khalil, 2015; Poniedziałek-Czajkowska et al., 2021).

The impaired production of pro/antiangiogenic factors, added to ROS stress, low activity of endothelial nitric oxide synthase (eNOS), low production of nitric oxide (NO) is characteristic of preeclampsia. This last gasotransmitter is a potent vasodilator, which is critical for appropriate trophoblast remodeling of spiral arteries (Poniedziałek-Czajkowska et al., 2021).

Sirtuin 1 (SIRT1) plays a critical role during pregnancy, via a stress-response and chromatin-silencing factor associated with a NAD-dependent histone deacetylase activity associated with DNA replication, DNA repair, an extension of life span, and reduction of apoptosis. Moreover, SIRT1 reduces the release of proinflammatory cytokines via inhibition of NF-kappa-B signaling (Chen et al., 2005; Abdelmohsen et al., 2007; Poniedziałek-Czajkowska et al., 2021). This signaling pathway is induced by AMP-activated protein kinase or AMPK (Yi et al., 2021). The SIRT1 expression in the placenta and the plasmatic concentration showed a low level in preeclamptic women at 20-25 weeks of gestation (Yin et al., 2017; Viana-Mattioli et al., 2020; Poniedziałek-Czajkowska et al., 2021).

The AMPK pathway is activated by increasing AMP levels and decreasing ATP levels (low energy). The AMPK is a heterotrimeric protein composed of αβγ subunits and expressed ubiquitously. The catalytic α subunit has two isoforms, but the α1 subunit (AMPK1) is the predominant subunit in Vascular smooth muscle cells (VSMCs) and endothelial cells, playing a role in vascular remodeling in atherosclerosis and pulmonary hypertension (Zhao et al., 2021). The AMPK1 gene is expressed in uterine arteries and placenta, and their expression increased during pregnancy exposed to hypoxia or models of preeclampsia (Skeffington et al., 2016), which is associated with the early onset of preeclampsia in humans (Liu et al., 2020). The pharmacological activation of AMPK1 can increase capacity to reduce the fetal restriction induced by hypoxia by an increase of uterine artery flow by approximately twofold (Lane et al., 2020), and these increases of blow flow can be stimulated by dependent via of NO (≈40%) and independent pathways (Skeffington et al., 2016), suggesting this protein plays a critical role in the vascular system of the placenta.

Another genetic component could potentially work during vascular pathologies such as preeclampsia. For example, an analysis of the transcript levels of 14,040 genes in the placenta from mothers with normotensive symptoms or hypertension during pregnancy was undertaken by Cox et al. (2019). The present re-analysis of data available in the GEO database[1] aims to find genes dysregulated at the vascular level that are associated with preeclampsia. Several pathways identified in the Kyoto Encyclopedia of Genes and Genomes (KEGG) can modulate vascular function. We identified the genes that were the most enriched and associated with the KEGG pathway as "negative regulation of vascular smooth muscle cell proliferation," "vasculature development," "vascular endothelial growth factor receptor signaling pathways," "vascular wound healing," "coronary vasculature development," "vasculogenesis," and "vascular endothelial growth factor receptor signaling pathways" see **Table 2**. Among the genes we identified, CITED2 plays a role in vasculogenesis, a critical gene in the cellular response to hypoxia, and showed the capacity to inhibit HIF1α activation and cellular response to hypoxia (Berlow et al., 2017). During preeclampsia, the activation of Endoplasmatic Reticulum stress induces the secretion of extracellular vesicles and the inhibition of CITED2 expression in the placenta (Collett et al., 2018). The CBP/p300-interacting transactivator, with glu/asp-rich c-terminal domain-2 or CITED2, plays a role in trophoblast differentiation and is expressed in vascular endothelial trophoblast cells. Their deletion induces placental malformation, decreasing placenta and embryo weight and reducing the number of placental malformation syncytiotrophoblasts, resulting in embryo death (Moreau et al., 2014; Imakawa et al., 2016). This suggests that these genes may be a new target of study. Similarly, another transcriptional via detected in the vasculogenesis pathway is oncogene JUNB (a subunit of AP1 factor), and their impaired expression in the placenta can elevate the risk of Preeclampsia. For example, the elevation of JUNB expression during pregnancy gives a high expression of Phosphatase and tensin homolog or PTEN protein and a reduction of approximately 50% of trophoblast invasiveness (Xue et al., 2020). Similarly, other studies in the cell line of the trophoblast suggest that elevated expression of JUNB can elevate the proliferation, migration, and stimulation of angiogenesis (Zou et al., 2018). In contrast, JUNB is downregulated in placenta-derived Mesenchymal Stromal Cells from the woman with preeclampsia (Nuzzo et al., 2017), suggesting this gene plays a critical role during pregnancy and preeclampsia.

A differentially expressed gene detected in **Table 2** is Cullin 7, or CUL7, a scaffolding protein expressed in all tissues and associated with ubiquitin ligase. Hypoxia during pathological pregnancies reduces CUL7 expression in the villous trophoblast and syncytiotrophoblast, inducing impaired placental development (Tsunematsu et al., 2006; Fahlbusch et al., 2012).

Another dysregulated gene detected in **Table 2** is the Vascular endothelial growth factor type A (VEGFA), which is critical during pregnancy for endothelial cell proliferation, migration, and angiogenesis. That is elevated in maternal serum in PE cases at 30 weeks and is sequestered during preeclampsia by excessive production of sFLT-1, resulting in endothelial dysfunction, suggesting an excess of VEGF production might play a role in preeclampsia by VEGF toxicity and stimulation of sFLT-1

[1]https://www.ncbi.nlm.nih.gov/gds

TABLE 2 | Functional annotation pathways modified by hypertension in placenta.

Negative Regulation of Vascular Smooth Muscle Cell Proliferation	Vasculature Development	Vascular Endothelial Growth Factor Receptor Signaling Pathway		Vascular Wound Healing	Coronary Vasculature Development	Vasculogenesis
GPER1 *G protein-coupled estrogen receptor 1*	**B9D1** *B9 domain containing 1*	**CRK** *CRK proto-oncogene, adaptor protein*	**NCKAP1L** *NCK associated protein 1 like*	**CD34** *CD34 molecule*	**SMAD6** *SMAD family member 6*	**CITED2** *Cbp/p300 interacting transactivator with Glu/Asp rich carboxy-terminal domain 2*
CNN1 *calponin 1*	**RIC8A** *RIC8 guanine nucleotide exchange factor A*	**FYN** *FYN proto-oncogene, Src family tyrosine kinase*	**SRC** *SRC proto-oncogene, non-receptor tyrosine kinase*	**ADIPOR2** *Adiponectin receptor 2*	**DCTN5** *dynactin subunit 5*	**JUNB** *JunB proto-oncogene, AP-1 transcription factor subunit*
CDKN1B *cyclin dependent kinase inhibitor 1B*	**ANP32B** *acidic nuclear phosphoprotein 32 family member B*	**ROCK2** *Rho associated coiled-coil containing protein kinase 2*	**WASF2** *WAS protein family member 2*	**HPSE** *heparanase*	**DNM2** *dynamin 2*	**SOX17** *SRY-box 17*
TGFB3 *transforming growth factor beta 3*	**CALCA** *calcitonin related polypeptide alpha*	**ACTG1** *actin gamma 1*	**CYFIP1** *cytoplasmic FMR1 interacting protein 1*	**NDNF** *neuron derived neurotrophic factor*	**GPC3** *glypican 3*	**CUL7** *cullin 7*
	ZFAND5 *zinc finger AN1-type containing 5*	**BMPR2** *bone morphogenetic protein receptor type 2*	**MAPK14** *mitogen-activated protein kinase 14*		**MEGF8** *multiple EGF like domains 8*	**GJC1** *gap junction protein gamma 1*
		CYBA *cytochrome b-245 alpha chain*	**MAPKAPK2** *mitogen-activated protein kinase-activated protein kinase 2*		**MYH10** *myosin heavy chain 10*	**GLMN** *glomulin, FKBP associated protein*
		ELMO1 engulfment and cell motility 1	***NCF4*** *neutrophil cytosolic factor 4*		**PLXND1** *plexin D1*	**HEG1** *heart development protein with EGF like domains 1*
		FOXC1 *forkhead box C1*	**VEGFA** *vascular endothelial growth factor A*		**PRICKLE1** *prickle planar cell polarity protein 1*	**VEGFA** *vascular endothelial growth factor A*
		HSPB1 *heat shock protein family B (small) member 1*	***VAV1*** *vav guanine nucleotide exchange factor 1*			
		ITGAV *integrin subunit alpha V*	**NCF1** *neutrophil cytosolic factor 1*			
		ITGB3 *integrin subunit beta 3*	**PIK3CB** *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta*			
		MAPK13 *mitogen-activated protein kinase 13*	**PIK3R1** *phosphoinositide-3-kinase regulatory subunit 1*			
		RAC1 *ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)*	***PTK2B*** *protein tyrosine kinase 2 beta*			
		SULF1 *sulfatase 1*				

The analysis of the transcript levels of 14,040 genes in the placenta from mothers with normal or hypertension during pregnancy, obtained by Cox et al. (2019). Re-analysis of data available in GEO (https://www.ncbi.nlm.nih.gov/gds) to find genes dysregulated at the vascular level, and perform functional analysis of differentially expressed genes characterized by gene ontology (GO) and pathway enrichment analyses (DAVID Bioinformatics Resources 6.8, NIAID/NIH). Our approximation gives co-expression network analysis by functional pathways that are modified in the placenta by hypertension and have a role in vascular health. The most enriched KEGG pathway saw more negative regulation of vascular smooth muscle cell proliferation, vasculature development, vascular endothelial growth factor receptor signaling pathways, vascular wound healing, coronary vasculature development, vasculogenesis, and vascular endothelial growth factor receptor signaling pathways.

production (Jena et al., 2020). Women with preeclampsia showed an elevated level of SRC protein, but the activation by the phosphorylation in the Tyr-416 residue was lowered, suggesting a low activation compared with normal women. Downstream genes activated by SRC, including ERK1/2, p38, and JNK showed low phosphorylation in preeclampsia, demonstrating the inactivation of SRC or c-SRC, which are critical for trophoblast development and differentiation. MAPK14 or p38-alpha is critical for trophoblast development and differentiation, and their activation stimulates the PPARγ pathway. This pathway induces the vascularization and expression of Syncytin-1, a critical element in placentation. In contrast, the human placenta from Preeclampsia showed a low level of expression of MAPK14-PPARγ- Syncytin-1 genes (Ruebner et al., 2012). Fyn is an oncogene that plays a role in the ERK via transduction, stimulating the expression of the KCa3.1 channel, an endothelial vasodilator, and inhibiting blood pressure increases during the pregnancy. However, another study observed the downregulation of this pathway during preeclampsia (Choi et al., 2019).

Another gene observed in **Table 2** is the Neutrophil cytosolic factor 4 or Ncf4, a subunit of NADPH oxidase, which is expressed in trophoblast cells after implantation (Gomes et al., 2012). This activity is the primary source of placental oxidative stress, which is a characteristic of preeclampsia. Similarly, another member detected is the subunit CYBA gene elevated in preeclampsia (Gomes et al., 2012; Trifonova et al., 2014), suggesting that the NADPH oxidase activity plays a critical role during the oxidative stress observed in the pregnancy. Moreover, elevated NADPH oxidase activity can modulate the production of sFlt-1 and PlGF, suggesting the critical role of this activity in developing preeclampsia (Hernandez et al., 2021).

The human placenta produces proteoglycans and the Glypican family are a member of these macromolecules. They are present in the Plasmatic Membrane by GPI anchor and can interact with VEGF. Glypican-3 (GPC3) modified their expression in pathological placentas (**Table 2**), playing an essential role in proliferation and differentiation and expressed in the human placenta. Low content is detected at the third trimester of gestation in samples of placenta and maternal serum from pregnancies diagnosed with Fetal Growth Restriction and preeclampsia (Chui et al., 2012; Gunatillake et al., 2019; Shimizu et al., 2019). Myosin heavy chain-10 or MYH10 modulates cell migration and invasion with preimplantation factor peptide or PIF to promote the implantation and remodeling of the uterine wall (Yang et al., 2018).

Another member of the Plasmatic Membrane observed in **Table 2** is the Plexin D1 or PLXND1, which plays a role in signaling endothelial cells and their impaired expression is associated with vascular disease, inducing atherosclerotic lesions by macrophages and inhibiting angiogenesis via stimulation of soluble Flt-1, an inhibitor of VEGF (Zhang et al., 2021). Similarly, we detected a differential expression in the pathological placenta of SMAD family member-6 or SMAD6 (see **Table 2**). When expressed postnatally this gene can modulate endothelial gene expression and participates in vascular development. Their mutation is associated with hypertension in children associated with renal arterial occlusive disease (Viering et al., 2020).

Preeclampsia is a two-stage disease with abnormal placentation and placental hypoxia by the impaired remodeling of the uterine wall. This affects the maternal endothelium and the production of Endothelial progenitor cells (EPC). The EPC circulating in maternal blood is characterized by CD34 antigen expression, and is used as a preeclampsia marker, related to vascular wound healing, and detected in pathological placenta (**Table 2**). Over 20 weeks gestation, women with preeclampsia showed a high level of Endothelial progenitor cells (CD34+) than the normotensive women (Brown et al., 2019). In contrast, in early pregnancy a low level of CD34 + cells have been observed (Laganà et al., 2017), suggesting that they are a marker dependent on gestational week and pathology during pregnancy.

Adiponectin receptor 2 (ADIPOR2) is a G protein-coupled receptor expressed in the embryo and placenta. ADIPOR2 induces a low proliferation via inactivation of the JNK pathway in the placenta and stimulates the lipid metabolism in the embryo. The human placenta is an independent source of Adiponectin, and their production in the placenta has proinflammatory effects and antiproliferative effects during the first trimester over trophoblast cells. However, several reports suggest a contradictory level of Adiponectin can be potentially produced by an adiponectin resistance state (Barbe et al., 2019).

Heparanase (HPSE) is a dysregulated gene observed in the placenta (**Table 2**) that plays a role in vascular wound healing, cleaving the heparan chain on the cell surface. Their products bind to sFLT-1, which promotes proliferation and invasion of trophoblast cells during early pregnancy (Che et al., 2018). However, their expression is elevated by hypoxia during preeclampsia, and this elevated activity stimulates the hypoxia-induced sFLT-1 release and inhibition of the proangiogenic function of VEGF (Ginath et al., 2015; Eddy et al., 2019). The bone morphogenetic protein receptor type-II or BMPR2 is a plasmatic membrane protein that transduces extracellular signals through the formation of heteromeric complexes, and their dysregulation plays a role during pulmonary hypertension vascular remodeling and endothelial dysfunction (Machado et al., 2003; D'Amico et al., 2018). We detected the dysregulated expression of BMPR2 in placentas from mothers with hypertension (see **Table 2**). Previous data show that BMPR2 and these ligands are critical for the maintenance of vascular development during pregnancy via VEGF production and invasion of the uterine wall and embryo placentation (Nagashima et al., 2013; You et al., 2021). Previous data suggest that Heparanase and BMPR2 can play a potential role during maternal hypertension in the placenta via inhibiting the proangiogenic effects of VEGF.

Another gene associated with the VEGF receptor signaling pathway (**Table 2**) is Rho-associated coiled-coil-containing protein kinase-2 or ROCK2. It is a serine/threonine kinase expressed at a high level in the placenta from preeclamptic women, inducing actin cytoskeleton rearrangement in the trophoblast cell and shedding of Syncytiotrophoblast macrovesicles and exosomes, accompanied with a decreased outgrowing microvilli (Han et al., 2016). Similarly, heat-shock 27-KD protein-1 or HSPB1 plays a role in the VEGF receptor signaling pathway and shows an impaired expression

in pathological placenta with hypertension (**Table 2**). Low expression of Het shock protein HSPB1 and HSP70 play a role in vascular alteration, and the umbilical artery flow modification detected in the placenta after premature birth (Dvorakova et al., 2017), which suggests that the relevance of different modulators can change the VEGF pathway in pathological placentas.

G protein-coupled estrogen receptor-1 (GPER1) proteins modify their expression in the placenta of mothers with hypertension (**Table 2**). They are a mediator of estrogen signaling and protect the fetus during maternal inflammation and are associated with negative regulation of vascular smooth muscle cell proliferation. For example, GPER1 protein can prevent the adverse effects of type-I Interferon during maternal infection (Harding et al., 2021). Moreover, the level of GPER1 in placentas from preeclampsia reduced by about 50%, a reduction that can partially be associated with estrogen treatment in trophoblast culture (Feng et al., 2017), which correlates with elevated apoptosis and minor cellular proliferation in the placenta from preeclampsia (Li et al., 2016). The functions of some of the genes detected in **Table 2** and their relation with the vascular disease require further study to better understand the role of these genes during hypertensive pathology during pregnancy and their role in the placenta.

Several reports suggest that melatonin plays a role as a protector agent during pregnancy for the mother, fetus, and placenta physiology (Dou et al., 2019; Nagasawa et al., 2021; Sun et al., 2021). Melatonin could directly stabilize blood pressure in pathological pregnancies via modifying previously described genes or new targets, which requires more studies to be conducted during gestational hypertension, preeclampsia, and other pathologies.

MELATONIN AND HYPERTENSION

Melatonin receptors are associated with the activation of two G-protein-coupled receptors named MT1 and MT2, which, via Gi-and Gq-receptor activation, lead to decreased levels of cAMP and increased levels of cytosolic calcium. Both receptors participate in the temporal synchronization of the circadian system and sleep quality (Jockers et al., 2016). Diurnal animals and humans have shown high blood pressure during daytime hours, and a dip of about 10-20% during darks hours such as human correlated melatonin secretion. Similarly, circadian rhythms have been observed for heart rate, which is abolished by the impaired secretion of the pineal hormone (Fabbian et al., 2013; Tabara et al., 2018). In contrast, the absence of circadian rhythms of blood pressure elevates the risk for cardiovascular morbidity/mortality by ventricular hypertrophy, renal dysfunction, remodeling of carotid structure, cerebrovascular accident, hypertension, and stroke (Baker and Kimpinski, 2018). The relevance of these circadian rhythms can be observed in the pharmacological treatment of hypertension with an angiotensin II receptor blocker, which is more effective during the higher production of melatonin hormone or night hours (Giles, 2006), suggesting the relevance of circadian rhythms and melatonin signaling for cardiovascular health.

A correlation between high blood pressure and arterial stiffness has been observed in patients, and the risk is higher when the disruption of the circadian rhythms of blood pressure is more severe. This severity is associated with the minor amplitude of the circadian rhythms or, eventually, a flattening of the circadian pattern, not showing a lower systolic or diastolic pressure during the night hours (Park et al., 2019). For example, after liver transplantation, about 90% of patients observed a chronodisruption of blood pressure oscillation, with about 55% showing an arrhythmic pattern and about 36% showed an inverted pattern. This chronodisruption has been associated with poor glomerular filtration of Cystatin-C and plasmatic accumulation (Hryniewiecka et al., 2018), the latter being a marker for robust kidney injury, systemic inflammation, and mortality (Hendrickson et al., 2020).

The melatonin receptor is present in several vascular tissues such as the Circle of Willis and vertebral arteries, the caudal artery, aorta, coronary arteries and carotids, cardiac ventricular wall, and systemic arteries, suggesting that melatonin plays a role in various cardiovascular diseases (Baker and Kimpinski, 2018; Prado et al., 2018). For example, a low level of melatonin is detected in Coronary heart disease (5-fold), elevating the risk of infarction and death. This can occur because the suppression of melatonin production induces vascular vasoconstriction and hypertension, and their supplementation reduces the blood pressure, inflammation, vascular infiltration of lymphocytes, aldosterone levels, and lowers the risk of deaths caused by myocardial infarction via reduction of oxidative stress (Baker and Kimpinski, 2018; Prado et al., 2018; Simko et al., 2018). Similarly, newborn sheep supplemented with melatonin showed reduced pulmonary arterial pressure. Moreover, the elevation of the vascular vasodilatation, which occurs via elevation of antioxidant capacity by stimulation of antioxidant activity SOD, CAT, GPx, causes induction of vasodilator genes and inhibition of vasoconstrictor gene response (Gonzaléz-Candia et al., 2020), suggesting the ubiquitous effects of this hormone in several vascular territories, lowering the risk of cardiovascular disease.

Interestingly, patients with pulmonary hypertension showed a low plasmatic level of melatonin and elevated levels of IL-1β. When analyzing animal models, supplementation with melatonin inhibits hypoxia-induced thickness and the remodeling of the pulmonary artery. It reduced the expression level of cytokine proinflammatory IL-1β in pulmonary tissue 3-fold and reduces macrophage activation (Zhang et al., 2020). A similar result was observed in gestational hypertension induced by L-NAME, melatonin supplementation can lower systolic blood pressure by about 10% and urine protein content by about 30%, increasing the antioxidant capacity of rats by about 28%, and lowering the sFlt-1 level circulation in about 29% of cases (Zuo and Jiang, 2020).

A study in patients with type 2 diabetes and hypertension demonstrated that about 30-32% of non-dipping people treated with 3-5 mg of melatonin saw a restoration of the dipping for systolic blood pressure, diastolic blood pressure, and mean arterial pressure during the dark hours, suggesting that melatonin could synchronize the circadian oscillation of blood pressure in about one-third of patients (Możdżan et al., 2014). A similar

observation was seen in animal models where melatonin administration reduced hypertension in animals with metabolic syndrome (Baker and Kimpinski, 2018). Moreover, melatonin has the dual capacity to modulate vascularization depending on the cellular condition. For example, in pathological tissues exposed to a lesion, melatonin induces angiogenesis, such as skin, bone, and gastric ulcers. This effect occurs because melatonin induces endothelial expression and secretion of VEGF, stimulating neovascularization (Ma et al., 2020).

Patients with dyslipidemia and atherosclerosis showed a low level of melatonin production, which lowers the plasmatic level of fibrinogen, FVIII, and leads to the inhibition of platelet aggregation (Otamas et al., 2020). A similar observation was made in postmenopausal women with prevalent hypertension, which showed a reduction of 26% in the urinary metabolite of melatonin 6-Sulfatoxy-Melatonin, and this chronic low-level melatonin elevates the risk of hypertension by about 17-23%. The risk was elevated by 60% when the patient reported using alcohol or medication to sleep (Pérez-Caraballo et al., 2018). A critical element for inducing vascular vasodilation is nitric oxide, which can be induced by melatonin, producing vasodilation, lowering blood pressure, and reducing Endothelin and Angiotensin II effects on humans umbilical vein endothelial cells (Baker and Kimpinski, 2018).

Hypertension and valvular dysfunction can induce heart failure and hypertrophy. The aortic constriction induces cardiac hypertrophy markers of natriuretic protein ANP, BNP, and β-MHC. However, these can be reverted by melatonin supplementation. Similarly, the apoptosis markers, caspase-3, cytochrome-c, and Bax, and the autophagy are lowered by melatonin treatment, suggesting the protective role of melatonin after aortic constriction. This inhibition of cardiac hypertrophy can occur due to the capacity of melatonin to stimulate the protein activation of p-mTOR, p-AKT, and the activation of the down pathways p-S6K and p-4E-BP1(Xu et al., 2020).

Several unknown pathways could potentially explain some of the effects of melatonin. For the functional analysis of the pathways correlated between melatonin and hypertension, we performed the search Analysis of Datasets of the GEO database[2]. Queries were performed using the "MELATONIN" keyword after a systematic SEARCH restricted to specific fields. We downloaded 39 experimental results for melatonin, and excluded the platforms without gene accession numbers, incomplete incoming information, cancer cells, transgenic animals, knockout, or experiments with modification of photoperiod. We then obtained experimental platforms "GSE92612" and "GSE169459" with vascular smooth muscle contraction pathways modified by melatonin. We observed 115 common genes, see **Table 3**. Furthermore, the genes that were combined and identified with Venn Diagram showed 35 common elements between "Hypertension" (**Table 1**) and "Melatonin," see **Table 4**. The differentially expressed genes obtained here showed several examples modified by melatonin over the vascular tone, which require further study into their potential role in hypertensive pathology during the pregnancy and their therapeutic role in protecting the placenta/fetus/mother from the adverse effects of hypertension.

TABLE 3 | Vascular smooth muscle contraction pathways modified by melatonin supplementation.

Differentially expressed genes associated with melatonin (*N* = 115 genes)					
ARAF	ADCY3	CALM2	MYLK2	PLA2G10	PRKACA
BRAF	ADCY4	CALM3	MYLK3	PLA2G12A	PRKACB
GNA11	ADCY5	CALML3	MYLK4	PLA2G12B	PRKACG
GNA12	ADCY6	CALML5	MYLK	PLCB1	PRKG1
GNA13	ADCY7	CALML6	NPR1	PLCB3	PPP1CA
GNAQ	ADCY8	EDNRA	NPR2	PLCB4	PPP1CB
GNAS	ADCY9	GUCY1A2	PLA2G1B	KCNMA1	PPP1CC
JMJD7-PLA2G4B	ADRA1A	GUCY1A3	PLA2G2A	KCNMB1	PPP1R14A
RAF1	ADRA1B	GUCY1B3	PLA2G2C	KCNMB2	PPP1R12A
ROCK1	ADRA1D	ITPR1	PLA2G2D	KCNMB3	PPP1R12B
ROCK2	AGTR1	ITPR2	PLA2G2E	KCNMB4	PPP1R12C
ARHGEF1	AVPR1A	ITPR3	PLA2G2F	KCNU1	RHOA
ARHGEF11	AVPR1B	MAPK1	PLA2G3	PTGIR	RAMP1
ARHGEF12	CALCRL	MAPK3	PLA2G4A	PRKCA	RAMP2
ACTA2	CACNA1C	MAP2K1	PLA2G4C	PRKCB	RAMP3
ACTG2	CACNA1D	MAP2K2	PLA2G4D	PRKCD	
ADORA2A	CACNA1F	MRVI1	PLA2G4E	PRKCE	
ADORA2B	CACNA1S	MYL6	PLA2G4F	PRKCH	
ADCY1	CALD1	MYL6B	PLA2G5	PRKCG	
ADCY2	CALM1	MYL9	PLA2G6	PRKCQ	

Functional analysis of pathways of melatonin by analysis of datasets from the GEO database (http://www.ncbi.nlm.Nih.gov/geo). The systematic search was restricted to the following specific fields: expression profiling by the array. In total, 39 experimental results for melatonin were downloaded, and we excluded the platforms without gene accession number, incomplete incoming information, cancer cells, transgenic animals, knockout, or experiments with modification of photoperiod. After this revision, we obtained experimental platforms "GSE92612" and "GSE169459," which were analyzed by GEO2R and selected vascular smooth muscle contraction pathways. Both experiments had 115 common genes.

TABLE 4 | Correlation of vascular smooth muscle contraction pathways modified by hypertension and melatonin supplementation.

Differentially expressed genes associated with hypertension *and melatonin (N = 35 genes)*			
ARAF	*GUCY1B3*	*PLA2G2A*	*PPP1CC*
GNAS	*ITPR1*	*PLA2G4A*	*RHOA*
RAF1	*ITPR2*	*PLA2G6*	*RAMP1*
ARHGEF11	*ITPR3*	*PLA2G2C*	*RAMP2*
ADCY2	*MAPK1*	*PLA2G5*	*RAMP3*
ADCY5	*MAP2K1*	*PLCB4*	
ADCY6	*MAP2K2*	*KCNMB4*	
ADRA1D	*MYLK2*	*PRKCD*	
CALM3	*NPR1*	*PRKCH*	
GUCY1A3	*NPR2*	*PRKCG*	

*Analysis by Venn Diagram from **Tables 1**, **3**. We identified 35 common elements between "Hypertension" and "Melatonin" associated with vascular smooth muscle contraction.*

Melatonin and Pregnancy

Several studies reported a relationship between hypertension and melatonin, inducing negative outputs during pregnancy via

[2]http://www.ncbi.nlm.Nih.gov/geo

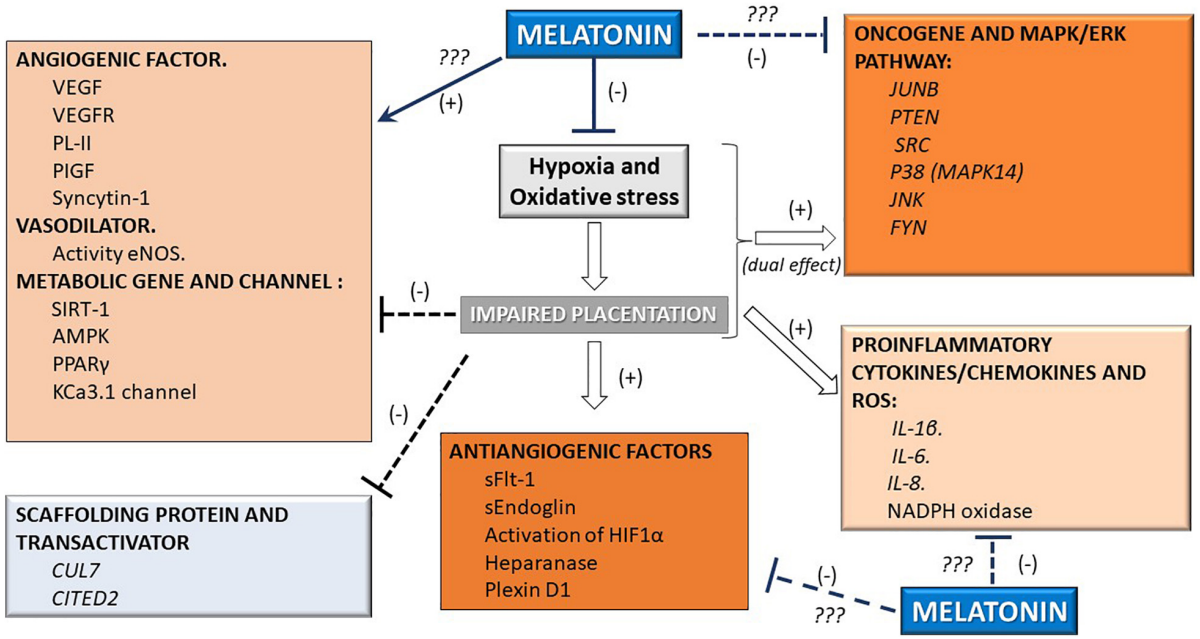

FIGURE 2 | Melatonin can modify molecular pathways in the placenta. Given the supraphysiological effect of the melatonin hormone, several pathways that affect vascular function are inhibited (-) or stimulated (+) in the placenta. These effects involve the hypoxia, pro/antiangiogenic, vasodilatory, metabolic, oncogenic, antioxidant, and proinflammatory pathways.

modifying the endothelial function, antiplatelet effects, vascular tone, vasoactive factor production, and oxidative stress. For example, during severe preeclampsia, melatonin production and the expression of MT1 and MT2 receptors are lower than in normal pregnancies. Moreover, the supplementation of melatonin to patients can delay the delivery and reduce oxidative stress and hypertension during pregnancy (de Chuffa et al., 2019). VEGF production during normal pregnancy is inhibited when the mother produces a low level of melatonin, increasing the risk of premature birth and abortion (Valenzuela et al., 2015; de Chuffa et al., 2019). During normal pregnancy, a progressive increase in melatonin levels is observed, and non-dipper blood pressure pregnant women with preeclampsia showed more severe hypertension correlated to a minor level of melatonin production during dark hours (Bouchlariotou et al., 2014). The placenta is an extra-pineal gland site for the synthesis of melatonin hormones, and placentas from preeclampsia have shown a low level of expression of the critical enzyme of melatonin synthesis in placenta AA-NAT and HIOMT. Interestingly, melatonin supplementation has antioxidant effects on the placenta and reduces the levels of sFlt1, Activin-A, and sEng, reducing trophoblastic debris from the early trimester placentae exposed to preeclamptic serum. The trophoblast mitochondria synthesize melatonin locally, protecting the mitochondrial and the respiratory function, a critical protagonist during placental hypoxia induced by preeclampsia (Langston-Cox et al., 2021). It prevents the oxidative stress of the placenta, inducing the activation of the antioxidant system via elevation of Nrf-2 translocation, a potent inductor of mitochondrial activity and biogenesis (Hobson et al., 2018). Melatonin supplementation during pregnancy in animal models increases umbilical blood (Thakor et al., 2010; Langston-Cox et al., 2021), protecting the endothelial function, repairing the endothelial monolayer, inhibiting vascular inflammation and VCAM-1 production in placentas obtained from preeclamptic women, and reducing blood pressure and sFLT-1, markers of vascular damage during preeclampsia (Hung et al., 2013; Reiter et al., 2017; Hobson et al., 2018; de Chuffa et al., 2019). Additionally, in women with early onset of preeclampsia, melatonin supplementation prolonged the interval from diagnosis to delivery in 6 days and required minor doses of antihypertensive treatment (Hobson et al., 2018), suggesting the partial inhibition of adverse effects of preeclampsia.

The umbilical blood sample collected at term from pregnancies affected by intrauterine growth restriction, or IUGRA, showed a lower level of melatonin circulation (∼50%). This reduction occurs parallel to the reduced circulatory levels of the angiogenic factor PIGF observed in the umbilical blood (Hobson et al., 2018; Berbets et al., 2020). The supplementation of melatonin in an animal model of gestational hypertension can lower the systolic blood pressure and urine protein content and ameliorate placental weight reduction. Moreover, it reduces the antiangiogenic production of sFLT-1, increases the proangiogenic factor PIGF, and increases the mother's antioxidant capacity (Zuo and Jiang, 2020). Similarly, melatonin reverted partially placental impaired perfusion, placental coagulation, and induced anti-inflammatory factors in mouse pregnancy associated with intrauterine inflammation-related oxidative stress (Lee et al., 2019). Reduction of oxidative stress and improvement of the placental perfusion induced by melatonin can occur

by an improved endothelial function via increased nucleus translocation of Nrf2 and elevation of endogenous antioxidant enzymes heme-oxygenase-1 (Hobson et al., 2018). These antecedents suggest the hormone melatonin's various actions on placental function and its potential role in modulating several modified pathways in pathological pregnancies (see **Figure 2**).

CONCLUSION

Melatonin hormone has antioxidant, homeostatic, and time-giving roles at the level of the vascular system. The temporal desynchronization of the vascular system by inhibition of melatonin production induces pathology such as hypertension. Melatonin supplementation shows a protective role over the vascular system, reverting elevation of blood pressure, oxidative stress, and antiangiogenic factors. During pregnancy, impaired production of melatonin can elevate the risk of poor fetal/placental development by preeclampsia, intrauterine growth restriction, and preterm birth. Melatonin can protect the pregnancy via stimulation of the antioxidant system, vascular factors such as VEGF, PIGF, and by inhibiting antiangiogenic factors such as sFLT-1 and sEng. Current evidence describes an elevation of melatonin production during pregnancy by the placenta, and we believe that local production is a keystone molecule in placental physiology. In this regard, we propose that melatonin plays a supraphysiological and dual role over placental physiology and could be the future for the protection and therapeutic application of vascular pathologies of pregnancies.

AUTHOR CONTRIBUTIONS

FV-M, CL, and GD contributed to conception and analysis. KJ-M and CL organized the database. FC-P and FV-M performed the bioinformatic analysis. FV-M wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Antiphospholipid Antibodies From Women With Pregnancy Morbidity and Vascular Thrombosis Induce Endothelial Mitochondrial Dysfunction, mTOR Activation, and Autophagy

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Antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombosis and pregnancy morbidity (PM) obstetric events together with persistent high titers of circulating antiphospholipid antibodies (aPL). Several mechanisms that explain the development of thrombosis and PM in APS include the association of aPL with alterations in the coagulation cascade and inflammatory events. Other mechanisms disturbing cellular homeostases, such as mitochondrial dysfunction, autophagy, and cell proliferation, have been described in other autoimmune diseases. Therefore, the objective of this study was to investigate the impact of aPL from different patient populations on endothelial cell mitochondrial function, activation of the mammalian target of rapamycin (mTOR) and autophagy pathways, and cellular growth. Using an *in vitro* model, human umbilical vein endothelial cells (HUVECs) were treated with polyclonal immunoglobulin G (IgG) purified from the serum of women with both PM and vascular thrombosis (PM/VT), with VT only (VT), or with PM and non-criteria aPL (seronegative-obstetric APS, SN-OAPS). We included IgG from women with PM without aPL (PM/aPL-) and healthy women with previous uncomplicated pregnancies (normal human serum, NHS) as control groups. Mitochondrial function, mTOR activation, autophagy, and cell proliferation were evaluated by Western blotting, flow cytometry, and functional assays. IgG from women with PM/VT increased HUVEC mitochondrial hyperpolarization and activation of the mTOR and autophagic pathways, while IgG from patients with VT induced endothelial autophagy and cell proliferation in the absence of elevated mTOR activity or mitochondrial dysfunction. IgG from the SN-OAPS patient

group had no effect on any of these HUVEC responses. In conclusion, aPL from women with PM and vascular events induce cellular stress evidenced by mitochondrial hyperpolarization and increased activation of the mTOR and autophagic pathways which may play a role in the pathogenesis of obstetric APS.

Keywords: antiphospholipid antibodies, antiphospholipid syndrome, endothelial cell, mitochondria, mTOR, autophagy

INTRODUCTION

Antiphospholipid syndrome (APS) is an autoimmune disease characterized by thrombosis and/or obstetric events together with persistent high titers of circulating antiphospholipid antibodies (aPL) (Miyakis et al., 2006). Thrombosis in APS can involve different components of the vascular bed in any tissue or organ, such as arteries (coronary artery disease, ischemic stroke, and transient ischemic attack), veins (deep venous thrombosis of lower limbs or pulmonary embolism), or small vessels (catastrophic APS with episodes of thrombosis in small vessels of multiple organs causing a systemic dysfunction). Pregnancy-related morbidity in APS may include early or late gestational losses, intrauterine growth restriction, fetal demise, preterm labor, or preeclampsia. In addition to the aforementioned clinical diagnostics defined by the Sapporo criteria (Miyakis et al., 2006), there are other clinical presentations not included. These manifestations can be hematologic (thrombocytopenia and hemolytic anemia), cardiac (heart valve disease), cutaneous (livedo reticularis), renal (nephropathy), or neurologic (cognitive dysfunction not associated with stroke) (Ziporen et al., 1996; Asherson et al., 2003; Garcia and Erkan, 2018; Turrent-Carriles et al., 2018; Kolitz et al., 2019). While APS is still considered a relatively rare disorder, our understanding of its diagnosis and management is continuously advancing (Tektonidou et al., 2019). Some recent studies have estimated that the prevalence of APS is 50 per 100,000 people, and the incidence is 2.1 per 100,000 person-years without differences between men and women (Duarte-Garcia et al., 2019). The estimated frequency of aPL in thrombotic complications was reported to be 9.5% for deep vein thrombosis, 11% for myocardial infarction, and 13.5% for stroke (Andreoli et al., 2013), the latter being more associated in patients under 50 years of age (Petri, 2000). The prevalence of obstetric complications was reported to be between 6 and 50% (Andreoli et al., 2013; Alijotas-Reig et al., 2015; Cervera et al., 2015; Esteve-Valverde et al., 2016).

The prevalence of aPL in the general population ranges between 1 and 5%. However, only a minority of these individuals will develop APS (Gomez-Puerta and Cervera, 2014). Pathological aPL are a heterogeneous population of autoantibodies mainly directed against phospholipid-binding proteins such as cardiolipin (CL) and/or β2-glycoprotein I (β2GPI) (Di Simone et al., 2007). Anti-CL and anti-β2GPI aPL, in combination with lupus anticoagulant (LA), constitute the current laboratory criteria for diagnosis. However, there is a group of aPL classified as non-criteria including antithrombin, anti-phosphatidylserine, and anti-phosphatidylethanolamine antibodies which are associated with APS (Bertolaccini et al., 2011). Several studies have described patients who lack the classical clinical manifestations of APS but who present consistently with high aPL positivity, and these cases are known as "non-criteria APS" (Tektonidou et al., 2019). In contrast, patients with clinical manifestations fulfilling APS classification criteria but who are consistently negative for aPL tests are classified as "Seronegative APS" patients (Hughes and Kamashta, 2003; Jara et al., 2017; Conti et al., 2019; Hughes and Khamashta, 2019).

Several studies have described the mechanisms by which aPL lead to prothrombotic and proinflammatory states. In endothelial cells, these mechanisms include alterations in the coagulation cascade and platelet activation; increased production of reactive oxygen species (ROS) and pro-inflammatory cytokines; and decreased nitric oxide production (Hidalgo, 2014; Mulla et al., 2018; Schreiber et al., 2018). Anti-β2GPI antibodies are recognized as the most pathogenic subset of aPL. Among them, the anti-domain I β2GPI antibodies have a strong correlation with thrombosis and with pregnancy morbidity (PM) (Iwaniec et al., 2017; Liu et al., 2020), which has been used as a predictor tool for patients with late PM (Chighizola et al., 2018). Studies have demonstrated that anti-β2GPI antibodies can activate receptors such as toll-like receptor (TLR) 4, TLR2, and Apolipoprotein E receptor E2 (APOER2) expressed on the surface of endothelial cells (Ramesh et al., 2011; Benhamou et al., 2014; Raschi et al., 2014), and this can lead to the activation of the nuclear factor kappa B (NFκB), p38 mitogen-activated protein kinase (p38 MAPK), and the phosphatidylinositol 3-kinase (PI3K) signaling pathways (Meroni et al., 2014; Chighizola et al., 2015). Another mechanism involved in APS pathophysiology is oxidative stress (Alves and Grima, 2003). It was recently demonstrated that monocytes and neutrophils, from patients with APS, display increased ROS production, increased expression of pro-inflammatory and prothrombotic molecules, and a loss of mitochondria function (Perez-Sanchez et al., 2012; Lopez-Pedrera et al., 2016). This mitochondrial dysfunction was also described in a mouse model of systemic lupus erythematosus (SLE) and was associated with activation of the PI3K pathway and mammalian target of rapamycin (mTOR) (Oaks et al., 2016), a kinase that modulates cellular growth, proliferation, and apoptosis (Magnuson et al., 2012). Activation of mTOR was also increased in renal endothelial cells from patients with APS samples (Canaud et al., 2014; Chighizola et al., 2015). In addition to cell growth and survival, mTOR activation is associated with anabolic mechanisms at the intracellular level (Magnuson et al., 2012), which leads to inhibition of catabolic processes like autophagy. However, the mTOR and autophagic pathways may both be activated under

conditions associated with oxidative stress and inflammation (Chen et al., 2011, 2016). Despite evidence of alterations in these pathways in other autoimmune diseases such as SLE (Lui et al., 2008; Oaks et al., 2016), less is known about the relationship between cellular metabolism and homeostasis in the context APS, and in particular, how aPL may disrupt the balance in endothelial cells. Therefore, the objective of this study was to investigate the impact of aPL on endothelial cell mitochondrial function, activation of the mTOR and autophagy pathways, and cellular growth.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from uncomplicated pregnancies based on a modified protocol by Jaffe et al. (1973) and as previously described (Velásquez et al., 2019; Gil-Villa et al., 2020). In brief, umbilical veins were perfused with 100 μg/ml type I collagenase (Invitrogen, Waltham, MA, United States) and incubated for 20 min at 37°C. Cells were recovered, and after centrifugation (50 *g* for 5 min), they were seeded in the endothelial cell growth medium (Promocell, Heidelberg, Germany) supplemented with 2% fetal bovine serum (FBS, Gibco, Waltham, MA, United States), 100 U/ml penicillin (Sigma Aldrich, Missouri, United States), 50 μg/ml gentamicin (Genfar, Bogotá, Colombia), and 0.25 μg/ml amphotericin B (Vitalis, Bogotá, Colombia). Isolated HUVECs were cultured in T75 cell culture flasks (Thermo Fisher Scientific, Waltham, MA, United States) at 37°C and 5% CO_2 until 100% confluent. The endothelial cell phenotype (CD31+) was confirmed by flow cytometry. All experiments were performed with different HUVEC clones from passages 1–3. All treatments were performed in Opti-MEM (Gibco) to keep the cells in FBS-free conditions.

Study Subjects

Patients were recruited from the Recurrent Pregnancy Loss Program of the Reproduction Group (University of Antioquia) and the Anticoagulation Clinic (San Vicente Fundación Hospital). Our Ethics Review Committee (Medical Investigations Institute from the School of Medicine, University of Antioquia) approved the collection of patient sera, and written consent was obtained from all participants. Women with clinical manifestations of APS were divided into the following three groups of study: women with clinical manifestations of PM and vascular thrombosis (PM/VT) or VT only (VT), positive for aPL as defined by the Sapporo criteria, and women with PM and positive for non-criteria aPL: seronegative-obstetric APS (SN-OAPS). Additionally, women with PM without aPL (PM/aPL-) and healthy women with previous uncomplicated pregnancies (normal human serum, NHS) were also included as control groups. Polyclonal immunoglobulin G (IgG) was purified from the serums of a total of 50 women included in this study for future cell treatments, and each group consisted of 10 patients. None of the patients were pregnant at the time the serum samples were obtained.

Antiphospholipid Antibodies

Anticardiolipin antibodies (aCL) were detected using a Commercial aCL ELISA Kit (BioSystems, Barcelona, Spain). Anti-β2GPI antibodies were detected using the AESKULISA β2-Glyco-GM Kit (Aesku Diagnostics, Wendelsheim, Germany) and Imtec β2GPI Kit (Human Biochemica und Diagnostica GmbH, Magdeburg, Germany). LA was detected in plasma samples following the recommendations of the Clinical and Laboratory Standards Institute (Ratzinger et al., 2017). APTT-SP (Instrumentation Laboratory, Bedford, MA, United States) was used to demonstrate the dependence of antibodies for phospholipids. Dilute Russell's viper venom time (dRVVT) screen and dRVVT confirmation (Instrumentation Laboratory) were used to detect LA. In addition, other non-criteria aPL were detected using an in-house ELISA standardized by the reproduction group based on the technique published by Kwak et al. (1992) and as previously described (Velásquez et al., 2019). In brief, U-bottom 96-well polystyrene microplates (Maxisorp Nunc^{TM}, Thermo Fisher Scientific) were covered with 30 μl of 50 μg/ml of the following phospholipids suspended in methanol: phosphatidylglycerol, phosphatidic acid, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol (Sigma-Aldrich, Saint Louis, MO, United States). The microplates were allowed to dry at 4°C overnight, then washed with 1 × phosphate buffered saline (PBS), and blocked with a buffer solution of PBS and 20% adult bovine serum (ABS, Gibco, United States) for 90 min at room temperature in the dark. After another wash with PBS, 50 μl of the sera or IgG of the patient were added in duplicate at a dilution of 1:50 or 250 μg/ml, respectively, in 20% ABS and incubated for 2 h in the dark. Then, the microplates were washed three times with PBS and incubated for 90 min with 50 μl of a 1:1,000 dilution of the antihuman IgG antibody conjugated to alkaline phosphatase (Thermo Fisher Scientific) and washed as above. Notably, 50 μl of the developer solution p-nitrophenyl phosphate (Sigma-Aldrich) was added at 1 mg/ml in a substrate solution (10% diethanolamine, 0.005% $MgCl_2$, and 0.02% sodium azide, pH = 9.8). The reaction was stopped with 50 μl of a 3 M NaOH solution. The optical density of each well was determined using an ELISA microplate reader (Multiskan FC^{TM}, Thermo Scientific) at a wavelength of 405–410 nm. In all assays, a blank with a developer solution and stop solution was included, as was a positive control and a negative control for each of the antigens. In addition, a non-specific binding control was included by placing each of the sera or IgG in a well without antigen, the value of which is subtracted from the average of the optical densities of the samples. Optical density values of the samples equal to or greater than 25% of the optical density of the positive control were considered positive. All patients were tested twice, at least 12 weeks apart.

To purify the total polyclonal IgG from the patient sera for the subsequent treatment of HUVECs, affinity chromatography was performed as previously described (Alvarez et al., 2017) using a MAb Trap^{TM} Kit (GE Healthcare, Chicago, IL, United States).

In brief, serum samples from each group were pooled, and the total protein was quantified. Pooled samples were centrifuged, filtered, and diluted 1:1 with a binding buffer to load samples of up to 25 mg. Samples were passed through a protein G Sepharose® prepacked column and eluted with the buffer supplied. The purified IgG was tested for endotoxins using the Limulus Amebocyte Lysate QCL-1000™ Kit (Lonza, Basilea, Swiss), and all preparations tested negative (data not shown). IgG integrity was also checked by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reduced and non-reduced conditions (data not shown).

Mitochondrial Membrane Potential and Lysosomal Acidification

Human umbilical vein endothelial cells were cultured in 24 well plates (5×10^4 cells/well) at 37°C and 5% CO_2. After 24 h, cells were incubated with 250 μg/ml IgG from all groups for a further 24 h. Then, cells were trypsinized, centrifuged, and stained either with 0.003 ng/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC-6) (Thermo Fisher Scientific) and 0.06 ng/ml propidium iodide (PI) (Sigma-Aldrich) for the mitochondrial membrane potential (MMP) test or with LysoTracker green DND-26 TM (Thermo Fisher Scientific) for the lysosomal acidification test. Then, flow cytometry was performed using an LSR Fortessa (Becton Dickinson, Franklin Lakes, NJ, United States), and at least 10,000 events per sample were acquired. The median fluorescence intensity (MFI) was recorded for DiOC-6 and LysoTracker green.

Western Blot

Whole-cell lysates were prepared from HUVECs grown in six well plates (1×10^5 cells/well) and stimulated with 250 μg/ml IgG for 1–24 h using 120 μl Laemmli sample buffer (Tris 1 M pH = 6.8, 20% SDS, 20% glycerol, 3.8% β-mercaptoethanol, and 8% bromophenol blue). Notably, 40 μl of protein extracts were resolved on 8–15% SDS-PAGE gels. To detect LC3-II/LC3-I, 30% glycerol was added to the gels. Separated proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Amresco, Solon, OH, United States). After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4°C with 1:1,000 dilution of the following primary antibodies in 10% bovine serum albumin (Thermo Fisher Scientific): rabbit antihuman phospho-RPS6 (Ser235/236), rabbit antihuman total RPS6, rabbit antihuman phospho-ULK1 (Ser757), rabbit antihuman total ULK1, rabbit antihuman LC3-I and LC3-II, and mouse antihuman α-tubulin (Cell Signaling Technology, Beverly, MA, United States). Membranes were washed and incubated with 1:2,000 or 1:5,000 dilution of goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase (Cell Signaling Technology). Peroxidase conjugated antibodies were detected by chemiluminescence using SuperSignal West Pico (Thermo Fisher Scientific). Images were captured using a G-Box photodocumentator (Syngene, Cambridge, England), and densitometry analysis was performed using Image J 1.51 (NIH, Bethesda, MA, United States).

Proliferation Assay

Human umbilical vein endothelial cells at 1×10^3 were seeded into 96 well microplates and cultured for 24 h. Then, cells were treated with 250 μg/ml IgG for a further 24 h. Cell proliferation was measured using the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology) following the instructions of the manufacturer. Optical densities were read at 450 nm on a Multiskan FC plate reader (Thermo Fisher Scientific). Optical density was directly proportional to the number of proliferating cells.

Statistical Analysis

All experiments were performed at least three times. Data are expressed as mean ± SE of the mean (SEM). Statistical significance was determined using one-way ANOVA with Holm-Šídák or Dunns post-test according to the data distribution, using GraphPad Prism 6™ (GraphPad Software Inc., La Joya, CA, United States).

RESULTS

Characteristics of Women Included in This Study

Women from the PM/VT and VT groups presented with clinical and laboratory features in keeping with the Sapporo criteria. The group of SN-OAPS women had a history of pregnancy-related morbidity, but they were only positive for the non-criteria aPL such as anti-phosphatidylglycerol and anti-phosphatidylethanolamine. The control groups, namely, PM/aPL- and healthy NHS women, were negative for all aPL laboratory tests. As expected, women from the PM/VT and VT presented with significantly higher levels of anti-β2GPI and anti-CL antibody titers when compared with the NHS and PM/aPL- groups. Data from the clinical and laboratory analyses are presented in **Table 1**.

Immunoglobulin G From Women With Pregnancy Morbidity/Vascular Thrombosis Increase Endothelial Cell Mitochondrial Membrane Potential

The balance between proton pumping by the electron transport chain and proton flow by complex V determines the MMP of a cell (Zorova et al., 2018; Morganti et al., 2019). High MMP levels result in the activation of the mitochondrial respiratory chain, which is an important source of ROS. Since high levels of ROS can cause cellular injury, maintaining elevated MMP levels is potentially harmful (Zorova et al., 2018). As shown in **Figure 1**, IgG from women in the PM/VT group significantly increased HUVEC MMP levels when compared with the NHS control. Levels of HUVEC MMP in response to IgG from patients with PM/aPL- were similar to that after exposure to the NHS control (**Figure 1**). No significant differences in HUVEC MMP levels were found after treatment with IgG from the PM/VT or SN-OAPS groups when compared with the NHS control group (**Figure 1**).

TABLE 1 | Clinical and laboratory features of the women included.

Parameter		NHS ($n = 10$)	PM/aPL- ($n = 10$)	PM/VT ($n = 10$)	SN-OAPS ($n = 10$)	VT ($n = 10$)
Age (mean of years ± SD)		37.6 ± 7.6	30.9 ± 5.5	36.2 ± 5.8	32.5 ± 4.6	32.8 ± 9.9
Previous fetal losses (mean and range)						
≤ 10 weeks of pregnancy		0	1.9 (1–3)	1.3 (1–5)	1.2 (1–2)	0
> 10 weeks of pregnancy		0	0.7 (1–3)	1.7 (1–5)	0.9 (1–4)	0
Preeclampsia < 34 weeks, n		0	0	6	0	0
Intrauterine growth restriction, n		0	0	2	0	0
Venous/arterial thrombosis, n		0	0	10	0	6
Associated systemic rheumatic autoimmune disease, n		0	0	1	0	4
Lupus anticoagulant (mean ± SD) [£]		1.06 ± 0.12	1.02 ± 0.08	2.43 ± 0.8 (+)[a,b]	1.06 ± 0.07	2.62 ± 0.55 (+)[a,b]
Positive patients for lupus anticoagulant, *n*		0	0	9	0	10
IgG aβ2GPI in serum (U/mL)[†]		2.29 ± 0.07	2.82 ± 0,43	73.4 ± 82 (+)[c,d]	3.16 ± 0.26	21.27 ± 30.5 (+)
Positive patients for IgG aβ2GPI, n		0	0	9	0	7
IgG anti-cardiolipin in serum (GPL/mL) [‡]		0	2.31 ± 0.45	113 ± 92.8 (+)[c,d]	2.05 ± 0.61	44.3 ± 43.9 (+)
Positive patients for IgG anti-cardiolipin		0	0	9	0	8
aβ2GPI in 250 μg/mL of IgG purified from sera (U/mL)§		0	0	62.75 (+)	0	27.21 (+)
Anti-cardiolipin in 250 μg/mL of IgG purified from sera (GPL/mL)‡		4.95	3.85	82.9 (+)	4.97	21.8 (+)
Serum other no-criteria IgG antiphospholipid antibodies (percentage of positivity of mean OD of patients/mean OD of positive controls)* [number of positive patients]	aPG	2.87 ± 5.07 [0]	3.62 ± 6.31 [0]	76.62 ± 57.89 (+) [8]	65.36 ± 57.0 (+) [7]	24.5 ± 32.91 (+) [3]
	aPA	4.38 ± 5.68 [0]	1.65 ± 1.79 [0]	62.91 ± 53.16 (+) [7]	26.36 ± 21.08 (+) [5]	46.79 ± 60.83 (+) [4]
	aPS	2.23 ± 2.56 [0]	6.53 ± 7.81 [0]	87.76 ± 112.23 (+) [6]	55.84 ± 52.07 (+) [7]	45.82 ± 41.24 (+) [4]
	aPE	1.28 ± 3.34 [0]	5.42 ± 6.22 [0]	28.10 ± 33.73 [4]	44.80 ± 36.39 (+) [6]	37.29 ± 52.97 (+) [5]
	aPI	5.51 ± 4.08 [0]	4.23 ± 5.23 [0]	56.19 ± 46.1 (+) [7]	48.39 ± 28.11 (+) [9]	42.88 ± 42.85 (+) [4]

*n, number of patients; (+) positive result; [a]p < 0.001 vs. NHS; [b]p < 0.001 vs. PM/aPL-; [c]p < 0.05 vs. NHS; [d]p < 0.05 vs. PM/aPL-; £ Positive > 1.2; [†]Positive at > 15 U/ml; [‡]Positive at > 10 GPL; [§] Positive at > 7 U/ml; * Positive at > 25%; aPG, anti-phosphatidylglycerol antibodies; aPA, anti-phosphatidic acid antibodies; aPS, anti-phosphatidyl serine antibodies, aPE, anti-phosphatidyl ethanolamine antibodies; aPI, anti-phosphatidyl inositol antibodies; aPL, antiphospholipid antibodies; NHS, normal human serum; PM, pregnancy morbidity; VT, vascular thrombosis; SN-OAPS, seronegative obstetric antiphospholipid syndrome; SD, standard deviation, IgG, Immunoglobulin G; aβ2GPI, anti-β2glicoprotein I; AL, lupus anticoagulant; GPL, standard units of IgG anticardiolipin.*

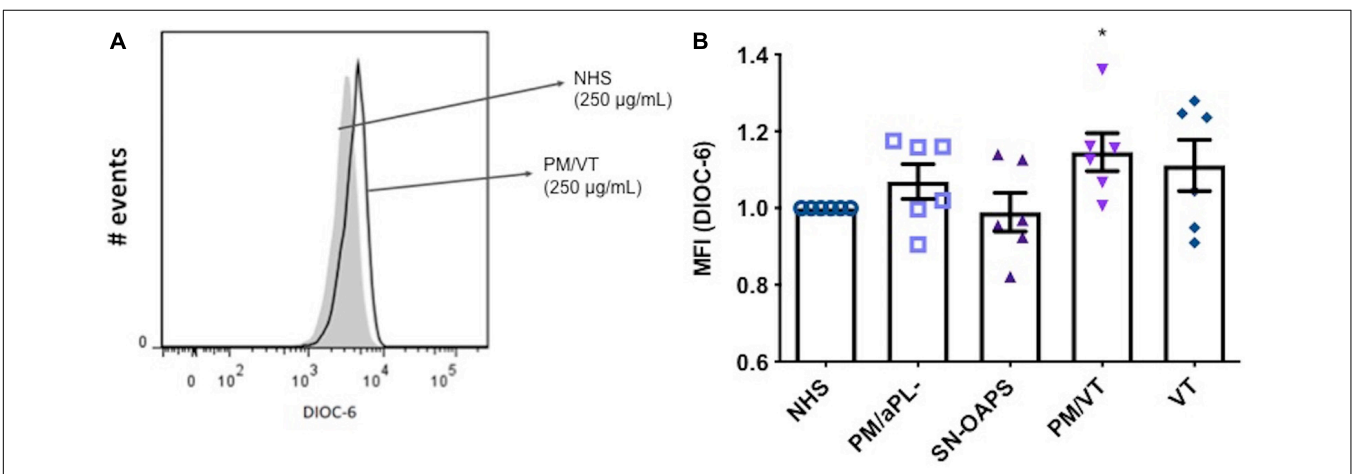

FIGURE 1 | Mitochondrial membrane potential (MMP) of endothelial cells is increased by antiphospholipid antibodies (aPL). Human umbilical vein endothelial cells (HUVECs) were stimulated with polyclonal immunoglobulin G (IgG) (250 μg/ml) from women with clinical features of antiphospholipid syndrome (APS) [seronegative-obstetric APS (SN-OAPS); pregnancy morbidity and vascular thrombosis (PM/VT); and VT] and control groups [normal human serum (NHS) and PM/aPL-]. MMP was measured by 3,3′-dihexyloxacarbocyanine iodide (DiOC-6) incorporation and flow cytometric analysis. **(A)** Representative histogram showing HUVECs treated with NHS IgG and PM/VT IgG. **(B)** The chart shows that treatment of HUVECs with PM/VT IgG induced a significant increase in MMP in comparison with NHS IgG. $n = 6$; $^*p < 0.05$ vs. NHS as determined by one-way ANOVA and Dunn's post-test.

Immunoglobulin G From Women With Pregnancy Morbidity/Vascular Thrombosis Activate the Mammalian Target of Rapamycin Pathway in Endothelial Cells

Perturbation of mitochondrial function and subsequent ROS production is known to influence the activation of the mTOR pathway, which in turn can impact the mitochondria through a retrograde signaling pathway (Kim et al., 2002; Sarbassov et al., 2005; Hopper et al., 2006; Schieke et al., 2006; Wullschleger et al., 2006). Therefore, we then evaluated the activity of the mTOR pathway by measuring phosphorylation of its effector protein RPS6. As a control, the mTOR inhibitor, rapamycin, reduced HUVEC phospho-RPS6 expression (**Supplementary Figure 1**). HUVEC expression levels of phospho-RPS6 after normalization to total RPS6 were similar in response to IgG from the NHS and PM/aPL- control groups (**Figure 2**). IgG from the PM/VT group significantly increased HUVEC phospho-RPS6 expression in comparison with IgG from the NHS control group (**Figure 2**). IgG from the VT and SN-OAPS groups had no significant effect on HUVEC phospho-RPS6 expression in comparison with either the NHS or the PM/aPL- controls (**Figure 2**).

Immunoglobulin G From Women With Pregnancy Morbidity/Vascular Thrombosis and Vascular Thrombosis Induces Autophagy in Endothelial Cells

To examine whether aPL have an effect on endothelial cell autophagy, we performed Western blots for the early autophagy marker ULK1 and the late autophagy marker LC3-II/LC3-I (Yu et al., 2018). As a control, the autophagy inducer, rapamycin, reduced HUVEC phospho-ULK1 and increased LC3-II/LC3-I levels (**Supplementary Figures 2, 3**). HUVEC expression levels of phospho-ULK1 after normalization to total ULK-1 were similar in response to IgG from the NHS and PM/aPL- control groups (**Figure 3A**). IgG from groups PM/VT and VT significantly reduced HUVEC ULK1 phosphorylation in comparison with the NHS control, while there was no significant difference with IgG from the SN-OASP group (**Figure 3A**). Similarly, the LC3-II-LC3-I ratio was significantly increased in HUVECs exposed to IgG from the PM/VT and VT groups when compared with the NHS control, while IgG from the SN-OASP group or the PM/aPL- control had no effect (**Figure 3B**). To further investigate autophagy at a functional level, HUVEC lysosomal acidification was examined (Yim and Mizushima, 2020) using LysoTracker®. As a positive control, rapamycin induced an increase in HUVEC lysosomal acidification, when compared to untreated cells (**Supplementary Figure 4**). As shown in **Figure 3C**, IgG from the VT group significantly increased HUVEC lysosomal acidification when compared with the NHS control. However, there was no evidence of increased HUVEC lysosomal acidification with IgG from the other groups (**Figure 3C**).

Immunoglobulin G From Women With Vascular Thrombosis Increases Endothelial Cell Proliferation

Since the main cellular processes controlled by the mTOR pathway are cell growth, proliferation, and survival, we evaluated the effect of IgG from the patient groups on endothelial cell proliferation using a BrdU incorporation assay. Levels of HUVEC cell proliferation were similar in the presence of IgG from the control groups NHS and PM/aPL-. IgG from individuals with VT significantly increased HUVEC proliferation in comparison with NHS only, while there was no effect by IgGs from the PM/VT or the SN-OAPS groups (**Figure 4**).

DISCUSSION

Endothelial cells are responsible for maintaining vascular homeostasis and play an important role in the development of thrombosis in patients with APS (Poredos and Jezovnik, 2018).

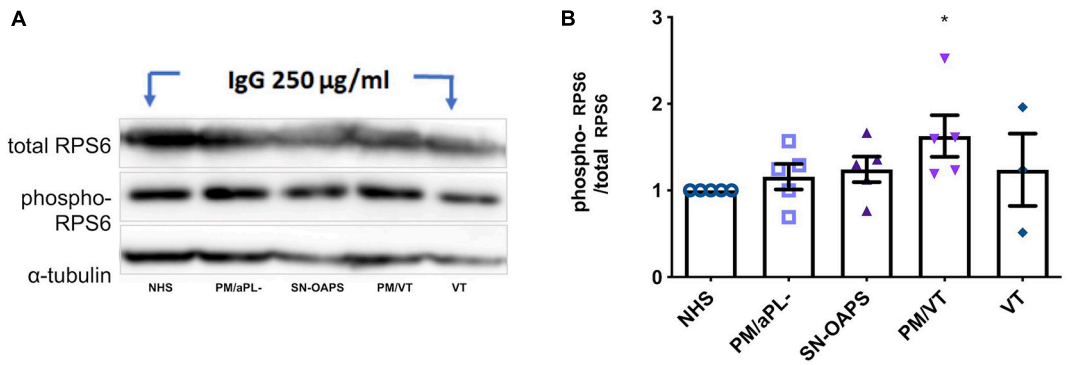

FIGURE 2 | Activation of mammalian target of rapamycin (mTOR) pathway is induced by aPL in endothelial cells. HUVECs were stimulated with 250 µg/ml polyclonal IgG from women with clinical features of APS and control groups. Protein expression of the total and phosphorylated forms of the effector ribosomal protein S6 (RPS6) was assessed by Western blot. **(A)** Representative blot. **(B)** Scatter plot shows the phospho/total RPS6 ratio. PM/VT IgG increased the RPS6 phosphorylation in comparison with the NHS IgG, which means that activates the mTOR pathway. Data are shown as mean ± SEM ($n = 5$; $^*p < 0.05$ vs. NHS from one-way ANOVA and Dunn's post-test).

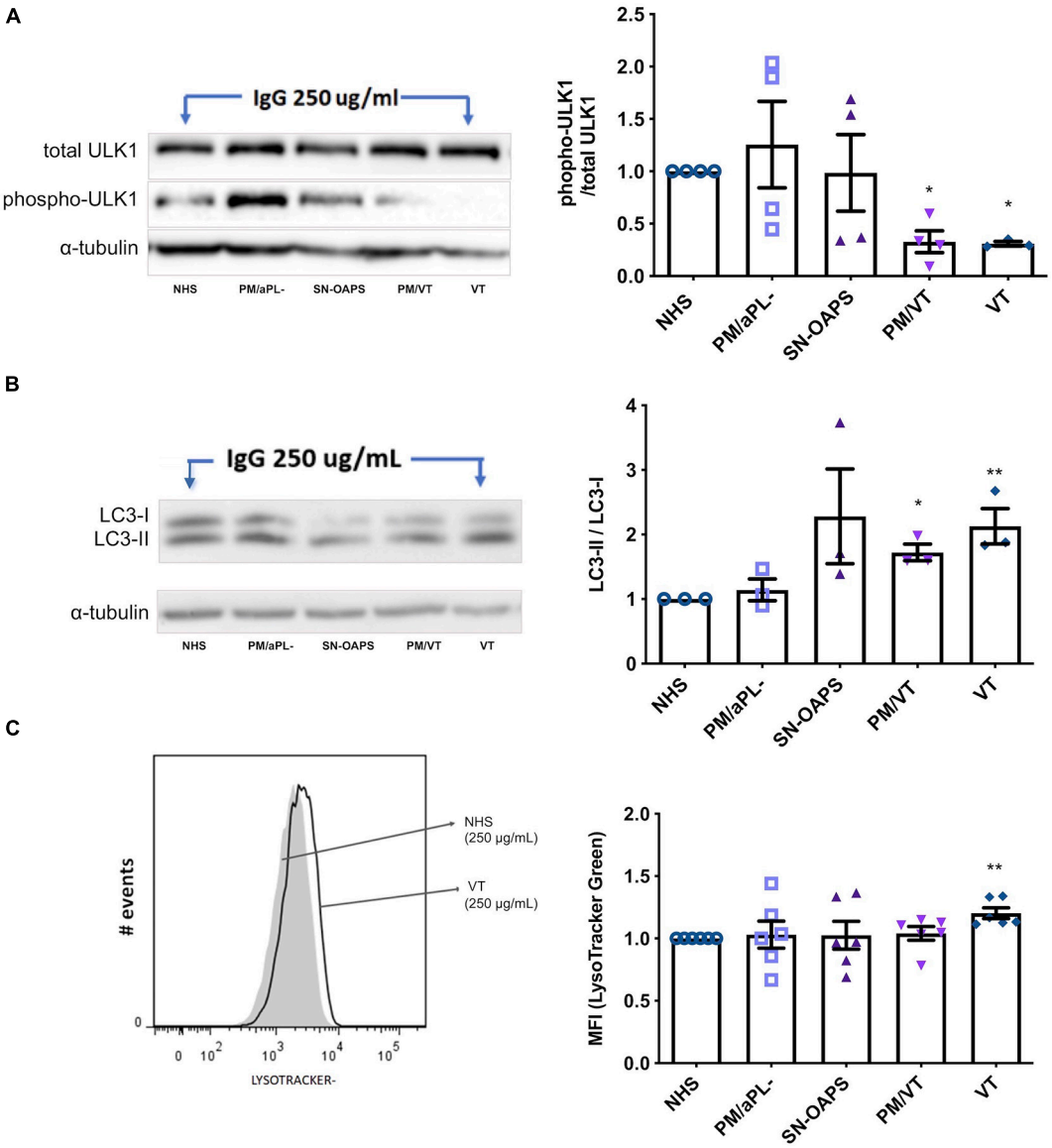

FIGURE 3 | Autophagy is activated by aPL in endothelial cells. HUVECs were stimulated with polyclonal IgG (250 µg/ml) from women with clinical features of APS (SN-OAPS; PM/VT; and VT) and control groups (NHS and PM/aPL-). **(A,B)** The early autophagy marker ULK1 and the late autophagy marker LC3-I/LC3-II were evaluated by Western blot. **(A)** Representative blot for phosphorylated and total ULK1. The chart shows the phospho/total ULK1 ratio. PM/VT and VT IgG significantly reduced ULK1 phosphorylation in comparison with NHS IgG ($n = 4$; $^*p < 0.05$ vs. NHS as determined by one-way ANOVA and Dunn's post-test). **(B)** Representative blot for LC3-I and LC3-II. The chart shows the LC3-II-LC3-I ratio. PM/VT and VT IgG significantly increased the LC3-II-LC3-I ratio in comparison with NHS IgG ($n = 3$; $^*p < 0.05$ and $^{**}p < 0.01$ vs. NHS as determined by one-way ANOVA and Holm-Sidak's post-test). **(C)** To evaluate autophagy at a functional level, lysosomal acidification was assessed using LisoTracker Green and flow cytometric analysis. Representative histogram showing HUVECs treated with NHS IgG and VT IgG. The chart shows the levels of LisoTracker Green as median fluorescence intensity (MFI) and that treatment of HUVECs with VT IgG significantly increased lysosomal acidification. $n = 6$; $^{**}p < 0.01$ vs. NHS as determined by one-way ANOVA and Dunn's post-test.

These cells are the target of several bioactive circulating factors that can cause a generalized dysfunction through deregulations in metabolism (Possomato-Vieira and Khalil, 2016; Bierhansl et al., 2017). MMP plays a key role in the maintenance of mitochondrial homeostasis (Zorova et al., 2018). MMP of a cell can change depending on the microenvironment, access to nutrients, cellular stress, and metabolic activity (Hirata and Sahai, 2017). aPL can induce a perturbation of the MMP in trophoblast cells, hepatocytes, and lymphocytes, leading to depolarization or hyperpolarization (Lai et al., 2015; Oaks et al., 2016; Alvarez et al., 2017). Since both depolarization and hyperpolarization involve dissociation of the electron transport chain and an increase of oxygen consumption, these conditions can be harmful to the cell through the induction of oxidative or reductive stress (Guo et al., 2011; Oaks et al., 2016; Zorova et al., 2018). We previously reported that serum from women

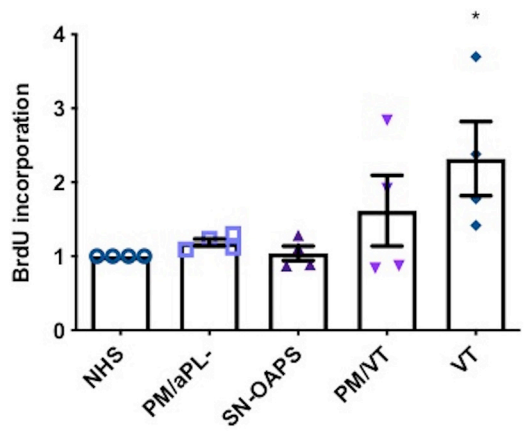

FIGURE 4 | Endothelial cell proliferation is increased by aPL. HUVECs were stimulated with polyclonal IgG (250 μg/ml) from women with clinical features of APS (SN-OAPS; PM/VT; and VT) and control groups (NHS and PM/aPL-). Cell proliferation was measured by BrdU incorporation. The chart shows that VT IgG significantly increased cell proliferation in comparison with NHS IgG. $n = 4$; $^*p < 0.05$ vs. NHS as determined by one-way ANOVA and Holm-Sidak's post-test.

with PM/VT increased the intracellular and mitochondrial production of ROS in HUVECs (Velásquez et al., 2019), the same group of patients whose IgGs induced the high MMP (hyperpolarization) in this study. Taken together, these findings suggest a mechanism by which aPL from women with PM/VT induced HUVEC oxidative stress. In fact, oxidative stress has been associated with endothelial cell dysfunction in preeclampsia, a clinical manifestation of obstetric APS (Rodríguez-Almaraz et al., 2018). Moreover, in the trophoblast, aPL recognizing CL and β2GPI bind to the mitochondria and induce ROS production (Zussman et al., 2020).

Oxidative cellular stress resulting from excessive metabolic ROS production can lead to the activation of rescue mechanisms such as autophagy. Autophagy is an intracellular degradation pathway that traffics substrates that could otherwise cause cytotoxicity (Chen et al., 2009; Ryter et al., 2019) through a catabolic system of double-membrane vesicles called autophagosomes, which are then fused with lysosomes (Bento et al., 2016; Qian et al., 2017). While some studies have reported altered autophagy in experimental models of APS (Mulla et al., 2018; Mu et al., 2020), less is known about how aPL impact endothelial cell autophagy. Endothelial cells control vascular homeostasis (Badimón and Martínez-González, 2002), and the mTOR pathway plays a major role in regulating cell metabolism, growth, and survival. There is also cross talk between the mTOR and autophagic pathways (Ryter et al., 2019). Classically, activation of the mTOR pathway has been associated with inhibition of autophagy through phosphorylation of ULK1 protein at Ser757 (Kim et al., 2011). However, in other contexts, such as tumor growth, there is coexistence between mTOR and autophagy activation. A similar behavior was observed in this study since the mTOR activation (determined by RPS6 phosphorylation) and concomitant autophagy activity (determined by a loss of ULK1 phosphorylation and increased LC3-II/LC3-I expression) were induced by IgG from patients with PM/VT and also IgG from patients with VT. In addition, IgG from women of the VT group induced lysosomal acidification, the ability of these aPL to trigger the final step of this degradative/recycling pathway.

Augmented endothelium cell survival has been associated with pathological conditions since it can lead to hyperplasia resulting in the occlusion of vessels and thus, generate a prothrombotic environment (Rajendran et al., 2013; Widlansky and Malik, 2015). aPL-induced proliferation was previously demonstrated in trophoblast cells (Alvarez et al., 2017). Another study showed increased cell proliferation in vascular endothelial cells exposed to aPL, which was associated with mTOR activation; and this activation was also observed in renal microvasculature of patients with APS nephropathy. In addition, patients who received rapamycin showed decreased vascular proliferation and no recurrence of vascular lesions (Canaud et al., 2014). This study highlights the relationship between cell survival pathways and cell proliferation under aPL conditions. Our findings of elevated cell proliferation in response to IgG from patients with VT only are, in part, in agreement with this report and indicate a link between the thrombotic features of that group of patients and the aPL-induced vascular effects.

CONCLUSION

This study aimed to establish a link between endothelial cell mitochondrial dysfunction, mTOR activation, and autophagy in the context of aPL, although it was performed in a descriptive way. Our findings have shown endothelial cell mitochondrial dysfunction in association with activation of the mTOR pathway and concurrent autophagic activity in response IgG from patients with PM/VT, while IgG from patients with VT only induced endothelial autophagy and cell proliferation in the absence of elevated mTOR activity or mitochondrial dysfunction. This underscores the heterogeneity of aPL. As expected, IgG from the control group PM/aPL- did not induce any responses in the endothelial cells. IgG from the patient group with clinical features of PM but non-criteria aPL (SN-OAPS) also did not induce any responses in the endothelial cells, thus revealing specific mechanisms triggered by the classical pathological aPL present in patients with PM/VT and VT. We previously showed several *in vitro* effects induced by aPL from women with PM/VT when compared with aPL from women with PM alone, suggesting that these aPL are distinct and could be triggering other pathways, which leads to different and/or more complex clinical manifestations (Alvarez et al., 2015, 2017). These subtle differences among the mechanisms triggered by aPL subpopulations have been suggested from prior studies (Ripoll et al., 2018; Alvarez et al., 2021). We also highlight that our cellular model was FBS-free, and since we did not performed any recombinant β2GPI addition, it is possible that cellular responses observed here were induced by aCL rather than aB2GPI antibodies. In conclusion, aPL from women with PM and vascular events induce cellular stress evidenced by

mitochondrial hyperpolarization and increased activation of the mTOR and autophagic pathways, which may play a role in the pathogenesis of obstetric APS. These pathways may provide us with pharmacological targets to study further since compounds such as rapamycin and chloroquine that inhibit mTOR and autophagy have been used in experimental models of APS (Xia et al., 2017; Liu et al., 2019; Miranda et al., 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee from the Medical Investigations Institute from the School of Medicine (University of Antioquia). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AA and AC responsible for obtaining the funds and designed this study. CRú and AC were responsible for the recruitment of patients. CRo and MV-B performed the experiments and analyzed the data. CRú performed and analyzed the hematological tests. CRo, AA, AC, MV, and VA wrote and performed a critical review of the manuscript. All the authors contributed to interpreting the results and revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.706743/full#supplementary-material

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Lactic Acid Transport Mediated by Aquaporin-9: Implications on the Pathophysiology of Preeclampsia

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Aquaporin-9 (AQP9) expression is significantly increased in preeclamptic placentas. Since feto-maternal water transfer is not altered in preeclampsia, the main role of AQP9 in human placenta is unclear. Given that AQP9 is also a metabolite channel, we aimed to evaluate the participation of AQP9 in lactate transfer across the human placenta. Explants from normal term placentas were cultured in low glucose medium with or without L-lactic acid and in the presence and absence of AQP9 blockers (0.3 mM $HgCl_2$ or 0.5 mM Phloretin). Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay and lactate dehydrogenase release. Apoptotic indexes were analyzed by Bax/Bcl-2 ratio and Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling assay. Heavy/large and light/small mitochondrial subpopulations were obtained by differential centrifugation, and AQP9 expression was detected by Western blot. We found that apoptosis was induced when placental explants were cultured in low glucose medium while the addition of L-lactic acid prevented cell death. In this condition, AQP9 blocking increased the apoptotic indexes. We also confirmed the presence of two mitochondrial subpopulations which exhibit different morphologic and metabolic states. Western blot revealed AQP9 expression only in the heavy/large mitochondrial subpopulation. This is the first report that shows that AQP9 is expressed in the heavy/large mitochondrial subpopulation of trophoblasts. Thus, AQP9 may mediate not only the lactic acid entrance into the cytosol but also into the mitochondria. Consequently, its lack of functionality in preeclamptic placentas may impair lactic acid utilization by the placenta, adversely affecting the survival of the trophoblast cells and enhancing the systemic endothelial dysfunction.

Keywords: AQP9, lactic acid transport, mitochondria, human placenta, preeclampsia

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INTRODUCTION

The normal growth and development of the fetus are sustained by the placenta. This ephemeral organ is more than just a selective barrier between the mother and the fetus. It is also a metabolically dynamic interface that uses part of the nutrient uptake to promote its own cellular growth (Vaughan and Fowden, 2016). In this context, emerging evidence shows that

the placenta may act as a sensor, detecting the availability of nutrients in the maternal circulation and adapting its metabolism to support fetal development (Díaz et al., 2014; Vaughan and Fowden, 2016).

Glucose is the primary substrate needed to meet the fetus and the placenta energy requirements (Baumann et al., 2002; Hay, 2006). The transfer of glucose across the placenta is mediated by specific Glucose transporters (GLUTs), GLUT1 being the most abundant isoform expressed at term (Illsley, 2000; Baumann et al., 2002). Besides, the level of lactic acid in fetal circulation is higher than in maternal circulation, suggesting that lactic acid could also serve as fuel for the fetus (Vaughan and Fowden, 2016).

Lactic acid exists in two isomeric forms: D-lactic acid and L-lactic acid. However, mammalian cells can only metabolize the L-lactate stereoisomer. Transcellular transfer of lactate is facilitated by a family of transmembrane proteins known as monocarboxylate transport system (MCT) that functions as a proton symport and is stereoselective for L-lactate (Halestrap, 2013; Iwanaga and Kishimoto, 2015). In the brain, it was reported that the transfer of monocarboxylates, such as lactate, may also be facilitated by aquaporin-9 (AQP9) (Badaut, 2010; Tescarollo et al., 2014). It was also found that lactate permeability increases with acidification suggesting that AQP9 may play a role as a channel for the protonated lactic acid form (Rambow et al., 2014). In addition, recent research proposes that lactate can also cross the mitochondrial membranes. In the mitochondria, lactate may be metabolized to pyruvate by the mitochondrial lactate dehydrogenase (LDH), leading to the formation of NADH. Thus, the production of NADH could scavenge reactive oxygen species (ROS) and protect cells from ROS-induced damage (Miki et al., 2013).

AQP9 belongs to a family of integral membrane proteins whose primary role is to facilitate transcellular water fluxes in response to osmotic gradients. In addition to water, AQP9 is also permeable to urea, glycerol, and monocarboxylic acids, like lactic acid, but it is impermeable to cyclic sugars as D-glucose (Tsukaguchi et al., 1998). Unlike MCTs, AQP9 can only transport the protonated form of monocarboxylates (Tsukaguchi et al., 1998; Rothert et al., 2017).

In normal human term placenta, MCT1 and MCT4 are localized on the basal membrane and the apical microvillus membrane of the syncytiotrophoblast cells (Settle et al., 2004; Nagai et al., 2010). MCT4 has a low affinity for lactate, playing a role in lactate export under conditions of high intracellular lactate (Halestrap, 2012). On the other hand, AQP9 is expressed in the apical membrane of the syncytiotrophoblast (Damiano et al., 2001) and the plasma membrane of the cytotrophoblast cells (Wang et al., 2004). However, at term, the cytotrophoblast layer is discontinuous and it does not restrict the transfer between the mother and the fetus.

In several placental disorders, such as preeclampsia, alterations in the formation of the syncytiotrophoblast may change the normal expression and function of many transport proteins and negatively impact the transfer of essential molecules, such as glucose, proteins, and oxygen (Brett et al., 2014).

In this regard, GLUT1 expression and function are downregulated in placentas from preeclamptic women (Lüscher et al., 2017), suggesting a reduction in glucose transport across the placenta. Additionally, a significant decrease was found in aerobic glycolysis in preeclamptic placentas (El-Bacha et al., 2019; Hu et al., 2021). Consequently, the trophoblast cells and the fetus might be driven to use an alternative source of energy like lactate.

Previously, we found that the molecular expression of AQP9 significantly increased in placentas from preeclamptic pregnant women (Damiano et al., 2006). However, functional experiments showed that water and monocarboxylate transport mediated by AQP9 were dramatically reduced (Damiano et al., 2006). Notwithstanding this, there is no evidence of alterations in the transcellular water transport between the mother and the fetus, suggesting that the main role of AQP9 in the human placenta is not related to water transport (Szpilbarg et al., 2018).

Given that AQP9 is also a metabolite channel, we proposed that this protein could be involved in placental energy metabolism. As a result, alterations in AQP9 may enhance syncytiotrophoblast stress, negatively affecting the survival of the cells. This feature may accelerate the release of apoptotic syncytial aggregates into maternal circulation potentially causing the damage of the endothelial cells.

However, the participation of AQP9 in the lactate transfer across the placenta was not investigated yet.

MATERIALS AND METHODS

Tissue Collection

This study was approved by the local ethics committee of the Hospital Nacional Dr. Prof. Alejandro Posadas and the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina [EXP-UBA: 45449/2017 Res(CD) No 2168/2017], and written consent was obtained from patients before the collection of the samples. Full-term normal placentas ($n = 16$) were obtained after cesarean section. All placentas were collected from healthy pregnant women who carried on an uncomplicated pregnancy and gave birth to a newborn without anomalies. Women who carried on multiple pregnancies, and those who had underlying maternal conditions, such as chronic kidney disease, chronic hypertension, liver disease, collagen vascular disease, diabetes, major fetal abnormalities, cardiovascular disease, and cancer, that could adversely affect the pregnancy were excluded. The clinical characteristics of the pregnant women are shown in **Table 1**.

Tissue Culture Conditions

The placentas were placed with the maternal side facing up and arbitrarily divided into four quadrants. Cotyledon fragments were isolated from different areas of each placenta midway between the chorionic and basal plate, using sterile dissection. After that, the decidua and basal plate were removed completely, and the placental tissue was thoroughly washed with saline solution to eliminate blood. Villous tissue was further dissected

TABLE 1 | Characteristics of pregnant women.

	Normal pregnant women
Number of pregnant women	16
Maternal age, *yr*	25.6 ± 1.7
Gestational age, *wk*	37.5 ± 0.1
Mean blood pressure, *mM Hg*	
Systolic	111 ± 3.7
Diastolic	64 ± 1.9
Proteinuria	negative
Body Mass Index (BM), *kg/m²*	24 ± 3
Birth weight, *g*	3450.5 ± 42.8
Fetal sex	
Male	9
Female	7
Placental weight, *g*	519.4 ± 14.3

Values are mean ± SD. All women were white Hispanic.

into explants of ∼50 mg and cultured as we previously described (Castro-Parodi et al., 2013). Briefly, explants were preincubated for 30 min in a free-serum medium to allow the tissue to recover from the isolation processes. Then, explants were placed into 24-well plates with low glucose Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc. BLR, Grand Island, NY, United States) and 100 IU/ml penicillin, 100 mg/ml streptomycin, 32 mg/ml gentamicin, and cultured at 37°C during 18 h. This medium contained 5 mM glucose and 1 mM sodium pyruvate, hereafter referred to as the low glucose medium. In some wells, this medium was supplemented with (a) 20 mM glucose (control situation), (b) 10 mM D-Lactic acid (Sigma-Aldrich Corp., San Luis, MO, United States), or (c) 10 mM L-Lactic acid (Sigma-Aldrich Corp., San Luis, MO, United States). D-Lactic acid is a stereoisomer of L-Lactic acid, which is not metabolized by mammalian cells. In all situations, osmolarity was adjusted by adding D-mannitol (Sigma-Aldrich Corp., San Luis, MO, United States).

In all the experimental conditions, explants were cultured in the presence and absence of 0.3 mM $HgCl_2$ (Sigma-Aldrich, San Louis, MO, United States), a nonselective inhibitor of AQPs, 0.5 mM Phloretin (Sigma-Aldrich, St. Louis, MO, United States) for specific blocking of AQP9 (Inuyama et al., 2002; Haddoub et al., 2009), and 50 mM alpha-cyano-4-hydroxycinnamic acid (CHC, Sigma-Aldrich, St. Louis, MO, United States) (Inuyama et al., 2002), a nonspecific inhibitor of MCTs. $HgCl_2$ stock solution was prepared in PBS, while Phloretin and CHC were diluted in DMSO. Vehicle controls were performed and no changes were observed compared with the untreated control (data not shown).

Experiments were conducted independently in triplicates and repeated at least three times.

The protein expression of AQP9 was tested by Western blot in the experimental conditions (Castro-Parodi et al., 2013).

MTT Incorporation

Viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich Corp., San Luis, MO, United States) assay as described previously (Castro-Parodi et al., 2013). After treatments, explants were incubated with 0.5 mg/ml MTT for 2 h at 37°C. After this time, each explant was put in another well containing 1 ml methanol to extract the formazan. Optical density was measured at 595 nm and values were relativized to the amount of total protein (Castro-Parodi et al., 2013).

LDH Release

The release of the cytosolic enzyme LDH in the extracellular environment due to the disruption of the plasma membrane may reflect that cells are dying by necrosis (Chan et al., 2013). LDH release was quantified in the culture medium using the colorimetric method described by Chan and coworkers (Chan et al., 2013). Briefly, LDH catalyzes the oxidation of lactate into pyruvate with the formation of NADH from NAD^+. Then, NADH is used in the conversion of the tetrazolium salt, 2-p-iodophenyl-3-p-nitrophenyl tetrazolium chloride, into a red formazan product. This reaction is catalyzed by the enzyme diaphorase. Formazan concentrations are directly proportional to the concentration of LDH. The optical density of the formazan product was measured at 492 nm and values were relativized to the amount of total protein.

Bax/Bcl-2 Ratio

After treatments, placental explants were processed as previously described (Szpilbarg et al., 2016). Briefly, explants were homogenized in lysis buffer containing 0.3 M NaCl, 25 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 1% Triton X-100, pH 7.4, supplemented with protease inhibitors: 0.2 mM PMSF, and 0.01 X Protease Inhibitor Cocktail Set III (Calbiochem®, EMD Millipore Corporation, Darmstadt, Germany). After centrifugation at 3100 *g* for 10 min, supernatants were collected and protein concentration was estimated by a BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific Inc. Waltham, MA, United States).

80 µg of protein was loaded into each lane, separated on a 12% polyacrylamide gel, and blotted into nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech Ltd., Pittsburgh, PA, United States). Membranes were blocked in 5% nonfat dry milk in Phosphate-buffered saline (PBS, pH 7.5) with 0.1% Tween 20 overnight at 4°C. Subsequently, they were immunoblotted with the primary anti-Bcl-2 (BD Biosciences Pharmingen, NJ, United States; 1:1000) and anti-Bax (Abcam, Cambridge, United Kingdom; 1:200) antibodies.

The antibodies were detected using horseradish peroxidase-linked goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, United States; 1:10,000) or anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, United States; 1:10,000) and visualized by chemiluminescence using the Enhanced Chemiluminescence Western Blotting Analysis System (ECL plus, Amersham Pharmacia Biotech Ltd., Pittsburgh, PA, United States) according to the manufacturer's instructions. Images were acquired using the ImageQuant LAS 500 chemiluminescence CCD camera (GE Healthcare, CA, United States) and the bands were quantified by the ImageJ 1.45 s software package. Detection of Bax and

Bcl-2 was performed separately on the same membrane. Equal loading of proteins in each lane was confirmed by staining the blot with Ponceau S (Sigma-Aldrich, San Louis, MO, United States). Results were expressed in arbitrary units.

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling Assay

Terminal Deoxynucleotidyltransferase-Mediated dUTP Nick-End Labeling (TUNEL) staining on histological sections was performed to identify apoptotic cells. The *in situ* Cell Death Detection Kit, Tetramethyl rhodamine (TMR) Red (Roche Applied Science, Indianapolis, IN, United States) assay was used according to the manufacturer's instructions. DAPI counterstaining was used to visualize cell nuclei. Both TMR- (red) and DAPI (blue) fluorescence were visualized by an epifluorescent microscope (Nikon, Eclipse E: 200).

Isolation of Mitochondrial Fractions and Detection of AQP9

Mitochondrial fraction was obtained from fresh placental tissue as previously described (Bustamante et al., 2014). Briefly, villous tissue free was dissected and homogenized in MSHE buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 5 mM Hepes, pH 7.4). The homogenate was centrifuged at 1,500 g for 10 min. The supernatant was recovered and centrifuged at 11,000 g for 10 min to sediment the total mitochondrial fraction. The supernatant was ultracentrifuged at 100,000 g for 60 min, resulting in a pellet designated as the microsomal fraction.

To separate the two subpopulations of mitochondria based on the specified sedimentation velocity, the total mitochondria fraction was centrifuged again at 4,000 g for 15 min. The obtained pellet corresponds to the "heavy/large" mitochondrial fraction. Following, the supernatant was centrifuged at 12,000 g for 15 min, obtaining a pellet described as "light/small" mitochondrial fraction (Bustamante et al., 2014). All centrifugations were carried out at 4°C. All the pellets were resuspended in MSHE buffer and protein concentration was determined as described above. Cytosolic and microsomal contaminations were assessed by determination of the specific activities of the lactic dehydrogenase and the antimycin A – insensitive nicotinamide adenine dinucleotide (NADH)-dependent cytochrome C reductase (Ramírez-Vélez et al., 2013; Bustamante et al., 2014).

Proteins were resolved by SDS-PAGE on a 12% gel, electrophoretically transferred to a nitrocellulose membrane. Membrane was probed with a polyclonal anti-AQP9 antibody (Alpha Diagnostic International Inc., San Antonio, TX, United States; 1:1,000) followed by incubation with a goat anti-rabbit immunoglobulin G (IgG; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, United States; 1:10,000) conjugated to peroxidase. Immunoreactivity was detected using the ECL plus (Amersham Pharmacia Biotech Ltd., Pittsburgh, PA, United States) as previously described. To confirm equal loading, each membrane was also stained with Ponceau S as a general protein marker (Lanoix et al., 2012; Szpilbarg and Damiano, 2017).

Characterization of Placental Mitochondrial Populations

Mitochondrial morphology of each subpopulation was analyzed by flow cytometry using a three-color FAC-SCAN cytometer equipped with a 15-mW air-cooled $\lambda = 488$-nm argon laser (Becton Dickinson, Franklin Lakes, NJ, United States) (Mattiasson et al., 2003). The mitochondrial size was determined by the Forward angle light scatter (FSC) of photodiode, and the response collected by an E-00 setting with logarithmic amplification gain of 5.39, and the mitochondrial structure was evaluated by the light scattered (SSC) at the perpendicular direction detected by a photomultiplier tube using a voltage of 578 and a linear amplification gain was adjusted to 4.3 (Bustamante et al., 2014).

To study the mitochondrial transmembrane potential ($\Delta\Psi m$), heavy and light mitochondria fractions were loaded with 30 nM of the potentiometric probe 3,3′-dihexyloxacarbocyanine Iodide (DiOC6, Thermo Fisher Scientific, Waltham, MA, United States) and evaluated by flow cytometry. The isolated mitochondrial fractions were either treated with 200 µm Ca^{2+}, to analyze calcium intake handling by mitochondria, 5 µm Carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP, Sigma-Aldrich, San Louis, MO, United States), as a positive control, or with PBS (control) for additional 5 min and immediately acquired by the cytometer. The working solutions of the probes were diluted in PBS. Fluorescence response was analyzed and differences were quantified in five independent experiments (Bustamante et al., 2014).

Transmission Electron Microscopy

Each mitochondrial fraction was washed with PBS and fixed in the glutaraldehyde fixation solution 2.5% in PBS for 4 h at 4°C. After washing twice, both fractions were fixed in 1% osmium tetroxide in PBS for 60 min at 4°C.

Subsequently, dehydration of the samples was carried out, using increasing concentrations of alcohol followed by acetone. Then, they were included in a water-soluble epoxy resin, Durcupan (Sigma-Aldrich, San Louis, MO, United States) at 60°C for 72 h to promote the polymerization. Once polymerized, 0.5 µm semi-fine cuts were made using an Ultramicrotome (Reichert Jung Ultracut E). The sections were mounted on slides, stained with toluidine blue, and observed under the light microscope.

The sections obtained in ultramicrotome were mounted on copper grids and were contrasted with uranyl acetate and lead citrate (Reynolds, 1963). Finally, they were observed under a transmission electron microscope (MET Zeiss 109) equipped with a digital camera (Gatan 1,000 W). The analysis of each mitochondrial fractions was based on mitochondrial inner membrane topology (Sun et al., 2007).

Statistical Analysis

The statistical analysis was conducted by GraphPad Prism 7.02 software (GraphPad Software, Inc. La Jolla, CA). All values were expressed as means ± SEM. The significance of the results was analyzed by Student's *t*-test, one-way ANOVA followed

by Bonferroni *post-hoc* test or two-way ANOVA where appropriate. Differences were considered significant at $p < 0.05$.

RESULTS

Effect of the Availability of Glucose and Lactate on the Viability of Trophoblast Cells Before and After the Blocking of AQP9

In order to evaluate the use of lactate as a glucose substitute, normal placental explants were cultured in (a) 25 mM glucose medium (control condition), (b) Low glucose medium, (c) Low glucose medium with D-Lactic acid, and (d) Low glucose medium with L-Lactic acid.

In all the tested conditions, the protein expression of AQP9 did not change (**Figure 1A**).

Explant viability, evaluated by MTT incorporation, decreased significantly in low glucose medium even in the presence of D-Lactic acid compared to those explants cultured in the control condition. Interestingly, when explants were cultured in low glucose medium supplemented with L-Lactic acid, MTT levels were similar to control (**Figure 1B**).

Even more, in this situation, the inhibition of MCTs did not affect cell viability, suggesting that L-lactic acid is passing through another transport protein. To investigate the contribution of AQP9 in the lactic acid transfer, we use $HgCl_2$ and Phloretin to block AQP9. We found that in explants cultured in low glucose medium supplemented with L-lactic acid, the blocking with $HgCl_2$ and phloretin significantly enhanced cell death. However, no difference was found between both inhibitions. In the other situations, cell death was not modified before and after the blocking of AQP9 (**Figure 1B**).

In addition, LDH release was analyzed to determine membrane integrity. Membrane leakage is usually associated with cell death by necrosis or late apoptosis. In all the situations tested, the release of LDH into the culture medium did not change, suggesting that cell death is not due to disruption of the plasma membrane (**Figure 1C**).

Effect of the Availability of Glucose and Lactate on the Apoptosis of Trophoblast Cells Before and After the Blocking of AQP9

To confirm the mechanism of cell death and the participation of AQP9 in the survival of the trophoblast cells, Bax and Bcl-2 expressions and the number of apoptotic nuclei were evaluated. Accordingly with the MTT incorporation, in explants cultured in both low glucose medium and low glucose medium supplemented with D-lactate, Bax/Bcl-2 ratio increased significantly compared to control and remained unaffected after the blocking of AQP9 (**Figure 2**). The addition of L-lactic acid to the low glucose medium prevented the Bax/Bcl-2 ratio increase (**Figure 2**). However, in this case, the inhibition of AQP9 resulted in an increased expression of the pro-apoptotic protein Bax and consequently in the Bax/Bcl-2 ratio (**Figure 2**).

Regarding the TUNEL assay, we observed the same behavior. In explants cultured in low glucose medium, the number of TUNEL positive cells was significantly higher than in those cultured in control condition while the addition of L-lactic acid abrogated the apoptosis. As expected, in the medium supplemented with L-lactic acid, the blocking of AQP9 gave rise to an increase in the number of apoptotic nuclei (**Figure 3**).

Isolation of Mitochondrial Fractions and Detection of AQP9

To investigate the subcellular localization of AQP9, total mitochondria fraction, and the heavy/large and light/small mitochondria subpopulations that coexist in the villous trophoblasts were isolated by differential centrifugation and characterized by flow cytometry and transmission electron microscopy (TEM). Cytosolic and microsomal contaminations were less than 1.8 and 2.3%, respectively.

In concordance with previous reports, we found that heavy/large particles showed high FSC while the light/small particles presented low FSC, both with similar SSC characteristics suggesting that despite the different sizes of the particles, the internal complexity was similar. The $\Delta\Psi m$ revealed that the light/small mitochondria fraction was depolarized with a level of polarization lower than the heavy/large mitochondrial fraction (**Table 2**). In the presence of the uncoupler FCCP and 200 µm Ca^{2+}, both fractions showed a decrease in their $\Delta\Psi m$.

TEM analysis of both mitochondrial fractions confirmed the presence of two phenotypes. Representative TEM micrographs are shown in **Figure 4A**. The analysis of the mitochondrial phenotype was based mainly on the inner membrane topology. The heavy fraction shows dense staining of the inner matrix with an intact outer membrane showing lamellar cristae. The light mitochondrial fraction exhibits an inner membrane enclosing separate vesicular matrix compartments or cristae and frequently swollen mitochondria with expanded matrix space, lack of staining of the matrix, and fragmented or disorganized phenotype.

Then, we explored the expression of AQP9 in trophoblast mitochondria. We showed evidence for the first time that this protein was found in the mitochondria fraction isolated from villous trophoblast cells (**Figure 4B**). As we previously reported, a band of 30 kDa corresponding to the AQP9 protein was also found in the microsomal fraction and no band was detected in the nuclear fraction (data not shown). We also analyzed the expression of AQP9 in the heavy and light mitochondrial subpopulations. We found that AQP9 is only present in the heavy mitochondria fraction (**Figure 4C**).

DISCUSSION

Previous studies have widely changed the conception that lactate is only a waste metabolic product of cell glycolytic metabolism (Gladden, 2004; Goodwin et al., 2015; Baltazar et al., 2020). In this regard, it is well accepted that glucose is metabolized by the placenta to generate lactic acid, which is the key fuel

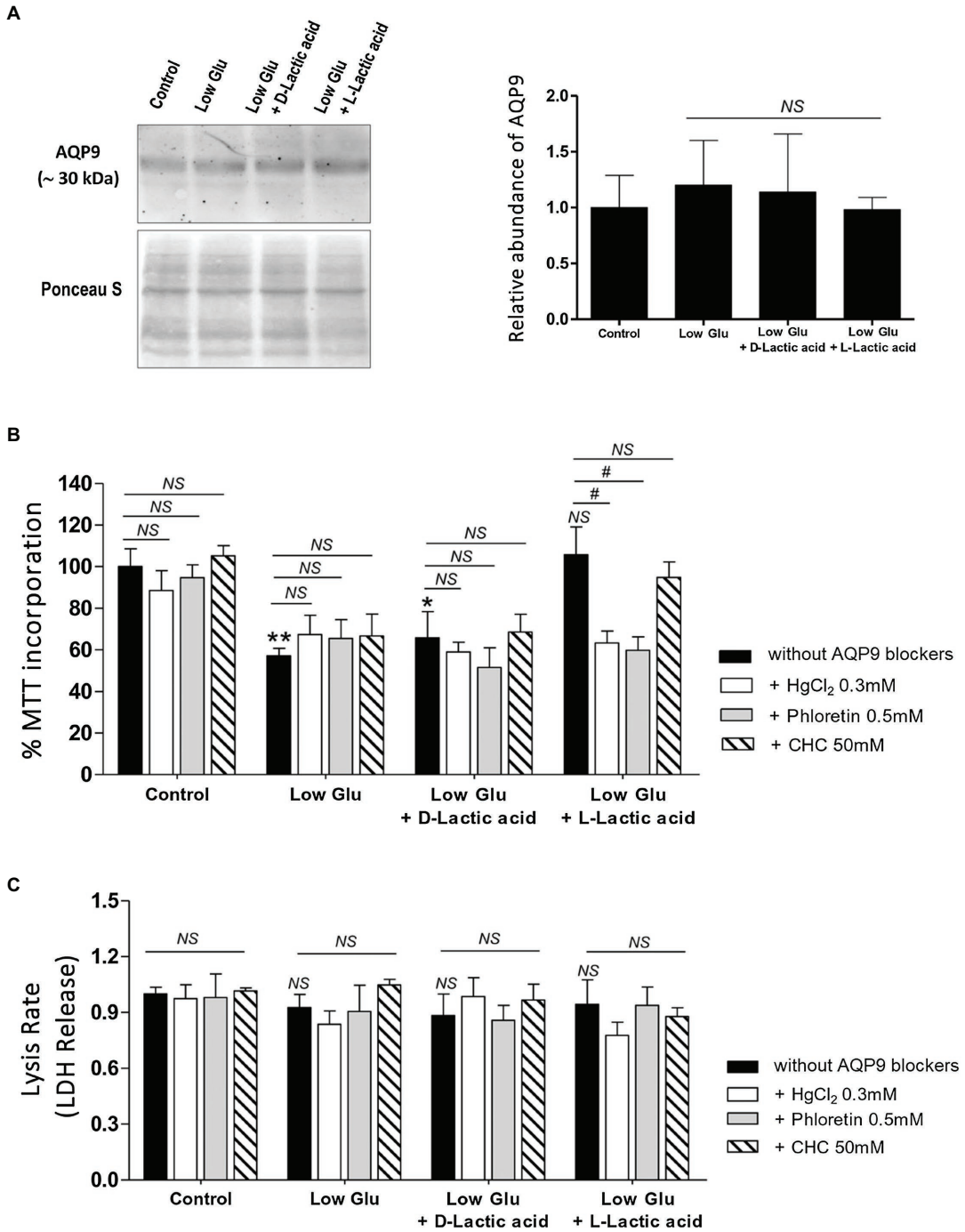

FIGURE 1 | Effect of the availability of glucose and lactate on the expression of Aquaporin-9 (AQP9) and the viability of explants. Explants were cultured in the different conditions: Control (25 mM glucose medium), Low Glucose medium (Low Glu, 5 mM glucose), Low Glucose medium with D-Lactic acid, and Low Glucose medium with L-Lactic acid. **(A)** Expression of AQP9. Representative Western blot for AQP9 protein expression in different culture conditions. Densitometry of immunoblot, the values were plotted as the relative abundance of AQP9 expression. ($n = 6$ placentas; NS=Non-significant). **(B)** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) incorporation. The viability was evaluated by MTT assay in placental explants cultured in the different conditions. CHC was used to block MTCs. The effect of 0.5 mM Phloretin or 0.3 mM $HgCl_2$ to block AQP9 was evaluated in all the treatments. All the experiments were independently conducted in triplicate. Data are expressed as means ± SEM. ($n = 6$ placentas; $^{*}p < 0.05$ and $^{**}p < 0.001$ compared to control without blockers, NS = Non-significant; and $^{\#}p < 0.05$ compared to low glucose medium with L-lactic acid without blockers). **(C)** Lactate dehydrogenase (LDH) release. LDH release assay was performed for determining the rate of cell death by necrosis in placental explants cultured in different conditions. CHC was used to block MTCs. The effect of 0.5 mM Phloretin or 0.3 mM $HgCl_2$ to block AQP9 was evaluated in all the treatments. The experiments were independently conducted in triplicate at least three times. Data are expressed as means ± SEM. ($n = 6$ placentas; NS = Non-significant). All the experiments were independently conducted in triplicates at least three times.

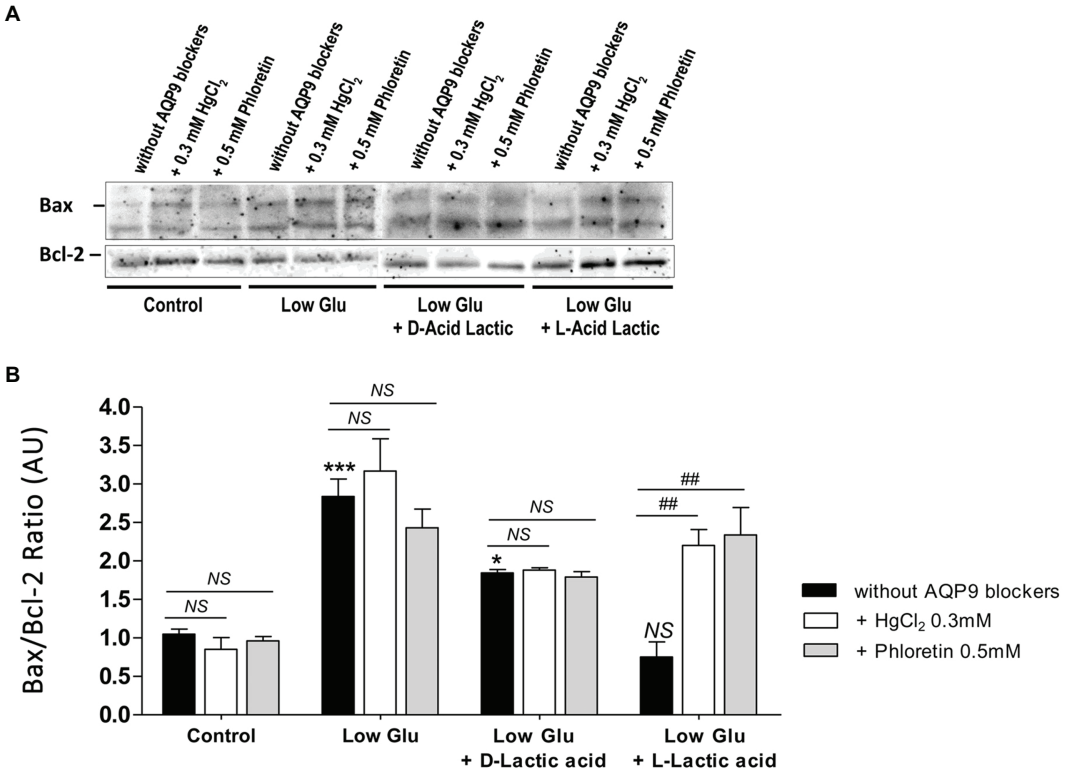

FIGURE 2 | Bax and Bcl-2 expression. **(A)** Representative Western Blot images and **(B)** densitometry for Bax/Bcl-2 ratio in placental explants cultured in the different conditions: Control (25 mM glucose medium), Low Glucose medium (Low Glu, 5 mM glucose), Low Glucose medium with D-Lactic acid, and Low Glucose medium with L-Lactic acid. The effect of 0.5 mM Phloretin or 0.3 mM $HgCl_2$ to block AQP9 was evaluated in all the treatments. Data are expressed as means ± SEM. $n = 6$ placentas. ($^{*}p < 0.05$, $^{***}p < 0.001$ compared to control without AQP9 blockers, NS = Non-significant; and $^{\#\#}p < 0.01$ compared to low glucose medium with L-lactic acid without AQP9 blockers).

for fetal growth (Baumann et al., 2002; Hay, 2005, 2006). In sheep, it was reported that lactate produced by the placenta represents almost 25% of fetal oxidative metabolism (Burd et al., 1975). Moreover, an association was found between reduced placental lactate transport to the fetus and fetal growth restriction (Settle et al., 2006). Nevertheless, it was not explored whether the placenta can use lactate to substitute glucose as an energy substrate when its availability in the maternal blood is reduced.

On the other hand, Miki and coworkers have reported that brain AQP9 can work with MCTs to transport lactate and speculated that it could have a role in energy metabolism and/or as a ROS scavenger (Miki et al., 2013; Akashi et al., 2015). Previously, we found that AQP9 expressed in the human placenta may not be only involved in water movement and homeostasis (Castro Parodi et al., 2011; Castro-Parodi et al., 2013). However, the role of AQP9 in the human placenta is still unknown.

In this work, we found that cell death was induced when placental explants were cultured in low glucose medium. There was no evidence of disruption of the plasma membrane, so cell death may take place by apoptosis. In this condition, the addition of L-lactic acid prevented cell death, and interestingly, the inhibition of MCTs did not affect cell viability revealing that another transport protein may be facilitating L-Lactic acid entry into the cell. On the other hand, the blocking of AQP9 led to an increase in both the pro-apoptotic protein Bax and the number of TUNEL positive nuclei in low glucose conditions.

Therefore, our findings suggest that trophoblasts can use L-lactic acid as an alternative source of energy when glucose availability is reduced by an AQP9-mediated mechanism.

It is well established that mitochondria orchestrate the process of life-and-death decisions of the cell (Can et al., 2014; Javadov et al., 2020; Marín et al., 2020).

In many tissues, it was proposed that lactate can enter the mitochondria and be metabolized to pyruvate by the mitochondrial LDH, whereas NAD^+ is reduced to NADH (Gladden, 2004; Passarella et al., 2014; Goodwin et al., 2015). Thus, NADH generated in the mitochondria can be re-oxidized to NAD^+ by the electron transport chain, while pyruvate can enter the tricarboxylic acid (TCA) cycle, which allows maintenance of the mitochondrial energy homeostatic cycle (Schurr and Gozal, 2012). Besides, NADH may act as a ROS scavenger. Thus, any alteration in NADH production may give rise to ROS accumulation triggering cell damage and finally leading to cell death (Miki et al., 2013). In this regard, there is considerable evidence that ROS promotes the apoptotic death

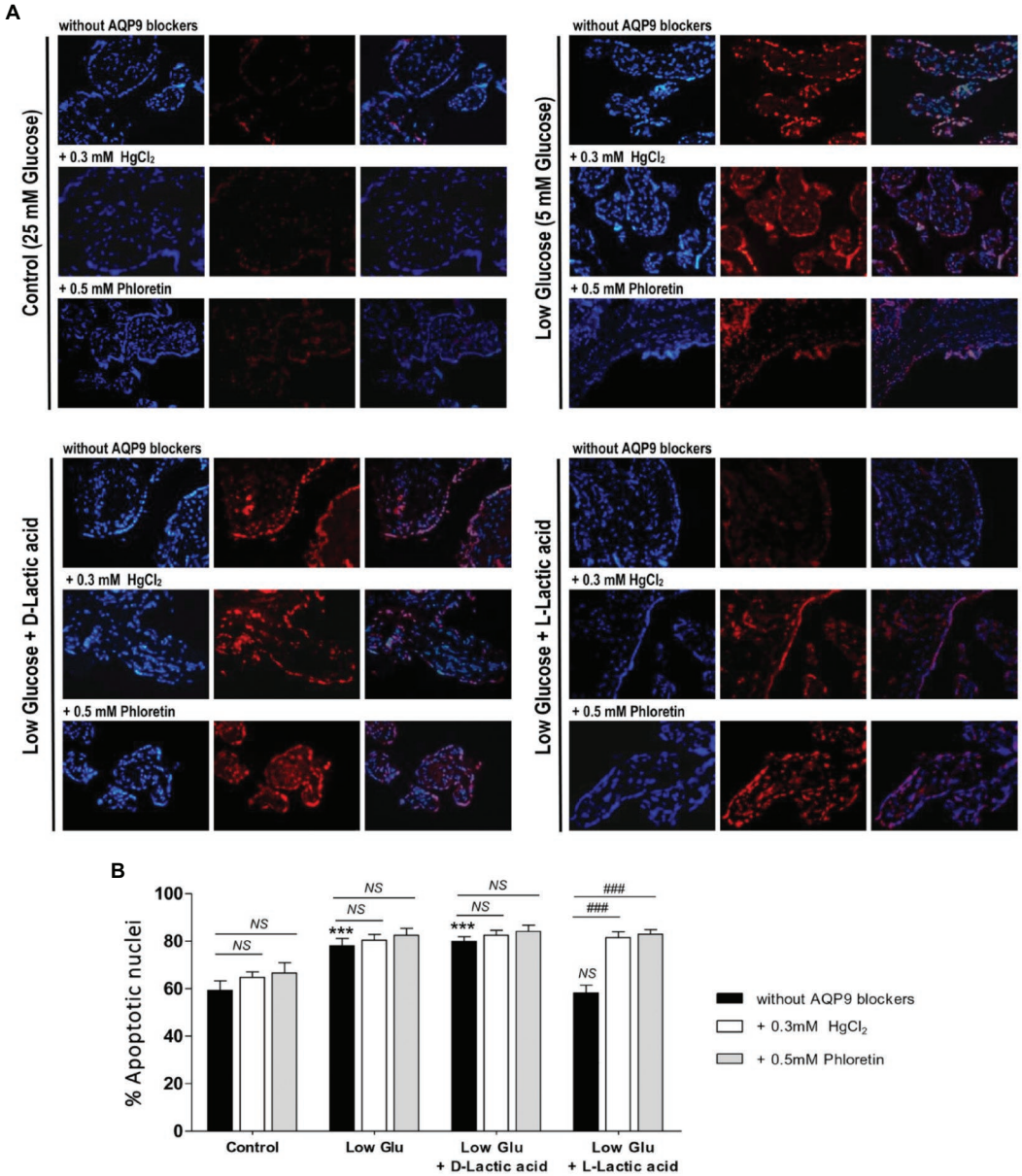

FIGURE 3 | TUNEL assay. **(A)** Representative images and **(B)** % of apoptotic nuclei for each treatment were shown. TUNEL positive cells (red); stroma nuclei stained with DAPI (blue). Image magnification: ×1,000. Data are expressed as means ± SEM. ($n = 6$ placentas; $^{***}p < 0.001$ compared to control without AQP9 blockers, NS = Non-significant; and $^{###}p < 0.001$ compared to low glucose medium with L-lactic acid without blockers).

TABLE 2 | Flow cytometry characterization and mitochondrial membrane potential in isolated mitochondrial fractions.

Mitochondrial Subpopulation	FSC	SSC	ΔΨm		
			Control	FCCP	Ca^{2+} 200 μm
Light/small	13.4 ± 1.8	22.3 ± 1.1	45%	24%	25%
Heavy/large	72.2 ± 1.3*	19.2 ± 1.8	64%	34%	29%

*$p < 0.05$ (Heavy/large mitochondrial fraction compared with Light/small mitochondrial fraction).
Values are expressed as means ± SEM, $n = 5$ placentas; % represents the percent respect to the total DIOC6 relative fluorescence intensity quantified in the homogenate. Experiments were independently conducted in triplicates at least three times. FSC = Forward angle light scatter, SSC = Side angle light scatter, ΔΨm = Mitochondrial transmembrane potential, and FCCP = Carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone.

of villous trophoblasts (Szpilbarg et al., 2016, 2018; Marín et al., 2020).

In the brain, it was reported that AQP9 also localizes in mitochondria (Amiry-Moghaddam et al., 2005), suggesting that mitochondrial AQP9 may function as a monocarboxylate channel working with MCT to transport lactate (Miki et al., 2013; Akashi et al., 2015).

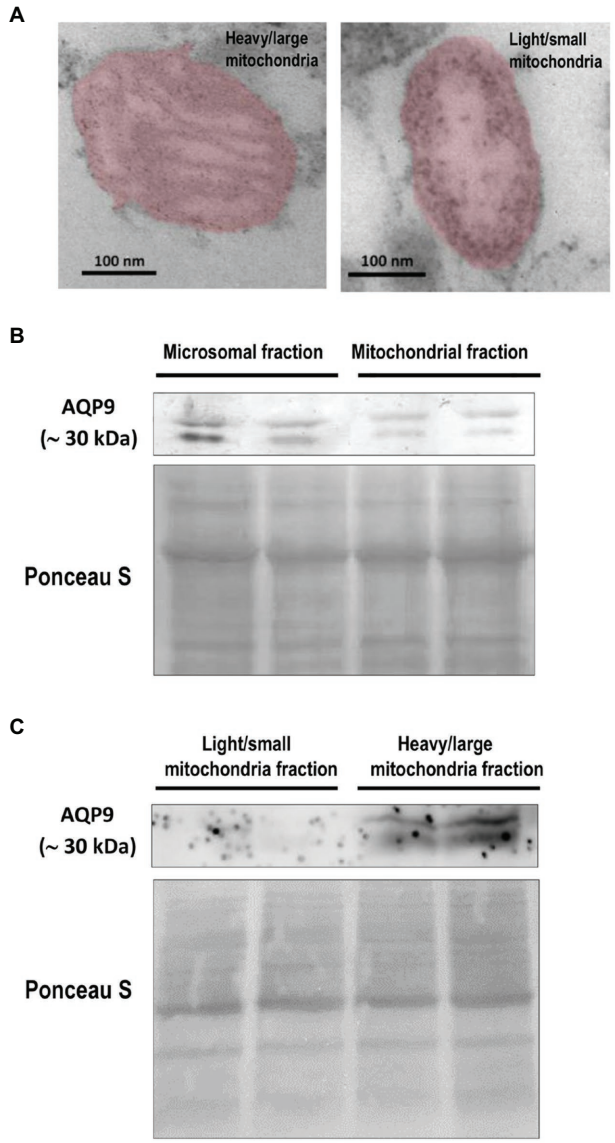

FIGURE 4 | Expression of AQP9 in trophoblast mitochondria. **(A)** Representative transmission electron microscope images of heavy/large and light/small mitochondria found in the isolated subpopulations. Individual mitochondria were falsely colored to aid in identification. The scale bar represents 100 nm and the magnification is 50,000 × ($n = 4$ placentas). **(B)** AQP9 expression in microsomal and mitochondrial fractions. Representative immunoblot revealed that AQP9 is expressed in both mitochondrial and microsomal fractions isolated from villous trophoblast cells. **(C)** AQP9 expression in heavy/large mitochondria subpopulation. Representative immunoblot showed the expression of AQP9 only in the heavy/large fraction ($n = 12$ placentas).

In human placenta, it is well documented that as cytotrophoblast cells differentiate into syncytiotrophoblast cells, trophoblast mitochondria undergo morphological and functional modifications. Previous reports showed that after *in vitro* fusion experiments, an accumulation of numerous small mitochondria was observed in the syncytial cells (Martinez et al., 1997). Thus, the "heavy" mitochondria fraction may be related to the cytotrophoblast while the "light" fraction may be linked to the syncytiotrophoblast (Martinez et al., 1997; Bustamante et al., 2014; Fisher et al., 2020). However, both mitochondria subpopulations may coexist in the syncytiotrophoblast.

In this context, we isolated both fractions and explored the expression of AQP9 in trophoblast mitochondria. According to previous work, we confirmed that the light/small mitochondria subpopulation is less polarized than the heavy one (Bustamante et al., 2014). Furthermore, the electron microscopy images showed well-defined differences not only in the mitochondrial morphology of each subpopulation but also in the inner membrane topology. Our results also revealed that AQP9 is present in the villous trophoblast mitochondria. Even more, this is the first report that shows evidence that this protein was only observed in the large/heavy mitochondria subpopulation.

Bustamante and coworkers have reported that the "heavy" fraction showed a better respiratory function, lower hydrogen peroxide production, lower mitochondrial P450, and higher cardiolipin concentration than the "light" fraction. In addition, they demonstrated that the "heavy" fraction expressed significant protein levels of p53, Bax, and cytochrome c compared with the "light" fraction (Bustamante et al., 2014). Based on these data, they suggested that the reduced oxygen consumption capacity, observed in the light fraction, may be related to a decrease in ATP production (Bustamante et al., 2014). Besides generating ATP, mitochondria also serve as local calcium (Ca^{2+}) buffers that tightly regulate intracellular Ca^{2+} levels (Haché et al., 2011). In this way, the electrochemical potential across mitochondria's inner membrane is used to sequester Ca^{2+}. Thus, a lower $\Delta\Psi m$ in the small/light fraction may reflect that calcium ions are dissipated more slowly across the inner mitochondrial membrane into the mitochondrial matrix, affecting the speed of electron transfer *via* the oxidative phosphorylation complexes and the citric acid cycle activity (Bertero and Maack, 2018).

All these differences suggest that both mitochondria fractions could be involved in different cellular processes. In this regard, Fisher and coworkers have recently proposed that the heavy mitochondrial subpopulation may participate in the physiological apoptotic mechanisms required for the normal differentiation and turnover of villous trophoblast cells (Fisher et al., 2020). Meanwhile, the light fraction may execute necrosis or autophagy (Fisher et al., 2020).

The evidence presented here supports the idea that in trophoblast cells, AQP9 may function as a lactate transporter together with MCTs. Since we found that AQP9 localized not only in the apical membrane (Damiano et al., 2001) but also in the mitochondria of the villous trophoblast cells, this protein may facilitate not only the lactic acid entrance into the cytosol but also into the mitochondria (**Figure 5A**). Along with this, we found that in a reduced glucose medium

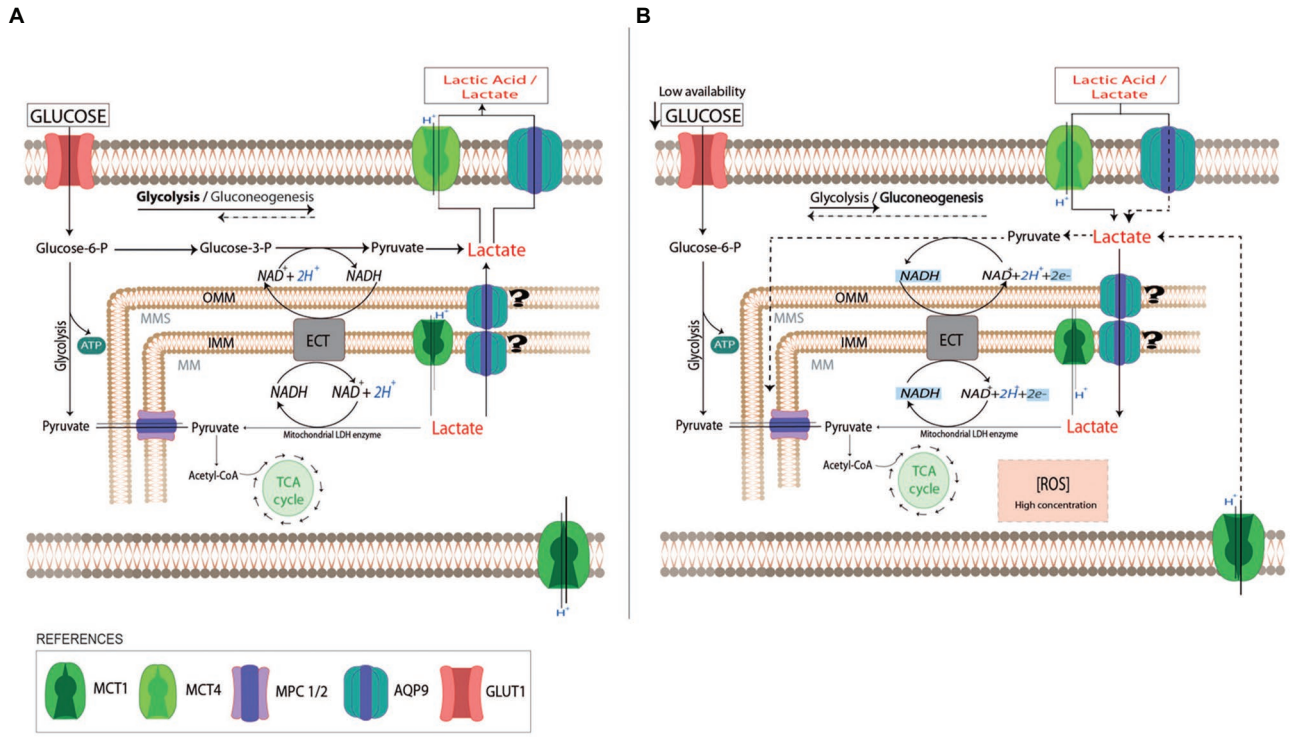

FIGURE 5 | Schematic representation of lactate use by trophoblast cells. In normal pregnancies, trophoblast cells use glucose as a fuel to support the placenta's cellular growth **(A)**. The excess of lactate due to fetal metabolism may be driven into the maternal circulation by monocarboxylate transports system (MCTs) and AQP9. When the availability of glucose is reduced **(B)**, trophoblast cells can use lactate as a carbon source substitute (dot lines). The uptake of lactate into the cytosol may be facilitated by AQP9 and MCTs localized in the plasma membrane. In the cytosol, lactate can be metabolized into pyruvate or it can pass across the external and inner mitochondria membranes by MCTs or AQP9. In the mitochondria matrix, lactate may be oxidated to pyruvate by a mitochondrial LDH while NAD^+ is reduced to NADH. The produced NADH may act as a reactive oxygen species scavenger. In preeclamptic placentas, AQP9 is not functional altering the use of lactate by the trophoblast cells. This may affect the mitochondria function, leading to the activation of the mitochondrial pathway of apoptosis. OMM, Outer Mitochondria membrane; IMM, Inner Mitochondria membrane; ECT, Electron transport chain; TCA cycle, tricarboxylic acid cycle; and MPC 1/2, Mitochondrial pyruvate carrier 1 and 2.

supplemented with L-Lactic acid, lactic acid cannot enter the cell when AQP9 is blocked, impairing mitochondrial function, resulting in the activation of the mitochondrial pathway of apoptosis. Therefore, it is possible that the ability of the villous trophoblast cells to better respond to the stress may be related to the content of heavy/large mitochondria with a functional AQP9.

It is well accepted that preeclampsia is usually associated with intermittent placental perfusion. Consequently, fluctuations in O_2 tension may enhance placental oxidative stress which has a critical role in exacerbating the villous trophoblast apoptosis (Hung et al., 2002; Hung and Burton, 2006; Marín et al., 2020). Considering the reduced GLUT1 expression and the decreased aerobic glycolysis observed in preeclamptic placentas, the concentrations of lactate in the placenta and the maternal blood might be increased. Although several reports have shown that plasma lactate levels are high in preeclampsia (Peguero et al., 2019), lactate concentrations are low in the placentas from preeclamptic women, suggesting that lactate cannot pass across the cell membrane of the trophoblasts.

Accordingly, we speculated that the increased oxidative stress observed in preeclampsia may impair AQP9 function as a lactate transporter. In this scenario, the lack of functionality of AQP9 may impair the lactic acid utilization by the placenta, promoting more accumulation of ROS and adversely affecting the survival of the trophoblast cells. This stress in the trophoblast cells may enhance the shedding of apoptotic aggregates into maternal circulation resulting in the systemic endothelial dysfunction that characterizes the maternal syndrome. Therefore, a non-functional AQP9 might be involved in the pathogenesis of preeclampsia.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Nacional Dr. Prof. Alejandro Posadas and Facultad de Farmacia y Bioquímica, Universidad de Buenos

Aires, Argentina [EXP-UBA: 45449/2017 Res(CD) N2168/2017]. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YM, LA, and JR carried out the experimental work and analysis of data. AC provided the placental tissues and discussed the results. NS and JB carried out data analysis and discussion and critically reviewed the manuscript. AD designed the study and wrote the manuscript. All authors contributed to the final version of the manuscript.

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Differences in Endothelial Activation and Dysfunction Induced by Antiphospholipid Antibodies Among Groups of Patients With Thrombotic, Refractory, and Non-refractory Antiphospholipid Syndrome

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Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by pregnancy morbidity or thrombosis and persistent antiphospholipid antibodies (aPL) that bind to the endothelium and induce endothelial activation, which is evidenced by the expression of adhesion molecules and the production of reactive oxygen species (ROS) and subsequent endothelial dysfunction marked by a decrease in the synthesis and release of nitric oxide (NO). These endothelial alterations are the key components for the development of severe pathological processes in APS. Patients with APS can be grouped according to the presence of other autoimmune diseases (secondary APS), thrombosis alone (thrombotic APS), pregnancy morbidity (obstetric APS), and refractoriness to conventional treatment regimens (refractory APS). Typically, patients with severe and refractory obstetric APS exhibit thrombosis and are classified as those having primary or secondary APS. The elucidation of the mechanisms underlying these alterations according to the different groups of patients with APS could help establish new therapies, particularly necessary for severe and refractory cases. Therefore, this study aimed to evaluate the differences in endothelial activation and dysfunction induced by aPL between patients with refractory obstetric APS and other APS clinical manifestations. Human umbilical vein endothelial cells (HUVECs) were stimulated with polyclonal immunoglobulin-G (IgG) from different groups of patients n = 21), including

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those with primary (VTI) and secondary thrombotic APS (VTII) and refractory primary (RI+), refractory secondary (RII+), and non-refractory primary (NR+) obstetric APS. All of them with thrombosis. The expression of adhesion molecules; the production of ROS, NO, vascular endothelial growth factor (VEGF), and endothelin-1; and the generation of microparticles were used to evaluate endothelial activation and dysfunction. VTI IgG induced the expression of adhesion molecules and the generation of microparticles and VEGF. RI+ IgG induced the expression of adhesion molecules and decreased NO production. RII+ IgG increased the production of microparticles, ROS, and endothelin-1 and reduced NO release. NR+ IgG increased the production of microparticles and endothelin-1 and decreased the production of VEGF and NO. These findings reveal differences in endothelial activation and dysfunction among groups of patients with APS, which should be considered in future studies to evaluate new therapies, especially in refractory cases.

Keywords: antiphospholipid syndrome, endothelial cells, endothelial activation and dysfunction, beta 2-glycoprotein I, immunoglobulin G, antiphosholipid syndrome

INTRODUCTION

The endothelium is a cell monolayer that lines the lumen of the lymphatic and blood vessels with paracrine, endocrine, and autocrine functions to control vascular remodeling and tone, blood flow, and leukocyte trafficking (Kruger-Genge et al., 2019). In diseases such as antiphospholipid syndrome (APS), endothelial activation is induced, thus producing proinflammatory and procoagulant molecules, leading to alterations in vascular tone, coagulability, and endothelial dysfunction (Liao, 2013; Corban et al., 2017; Miranda et al., 2019). APS is an autoimmune disease characterized by clinical manifestations of thrombosis or pregnancy morbidity and persistent antiphospholipid antibodies (aPL), including lupus anticoagulant (LA), anti-cardiolipin antibodies (aCL), and anti-β2-glycoprotein-I (aβ2GPI; Miyakis et al., 2006). The association between endothelial activation and dysfunction and the clinical manifestations of patients with APS is unclear (Velásquez et al., 2018).

Patients with APS present different clinical manifestations, aPL profile immunoglobulin (Ig) isotype, titers, and medication response. Patients with obstetric APS who only have pregnancy morbidity and are repeatedly positive for at least one of the aPL have a successful pregnancy in 75% of the cases if they receive heparin and aspirin (ASA) as standard treatment (Schreiber and Hunt, 2019). Contrarily, patients with obstetric APS who have pregnancy morbidity plus thrombosis and high aPL titers, particularly those with triple aPL positivity, have an ineffective standard of care, but the addition of hydroxychloroquine (HCQ) improves the gestational outcome (De Carolis et al., 2017; Ruffatti et al., 2017; Mekinian et al., 2018). aPL in these patients lead to endothelial activation and dysfunction, which deteriorate vascular relaxation through diverse mechanisms, including (1) the production of reactive oxygen species (ROS); (2) decreased bioavailability of nitric oxide (NO); (3) enhanced synthesis of vasoconstrictor factors, such as endothelin-1; (4) adhesion molecule synthesis; and (5) the release of endothelial microparticles (Mayer-Pickel et al., 2016; Engel et al., 2017; Sacharidou et al., 2018; Miranda et al., 2019; Velasquez et al., 2019; Alvarez et al., 2021). These pathological effects are induced by beta 2-glycoprotein-I (β2GPI) bound to the endothelium, but this mechanism is unclear.

Endothelial dysfunction is marked by a decrease in the synthesis and release of NO from the endothelium (Cyr et al., 2020). NO downregulates the interaction with leukocytes via a decreased expression of adhesion molecules (Gao et al., 2017). However, adhesion molecules and ROS are upregulated under endothelial dysfunction, generating a vicious circle of deterioration in NO availability (Forstermann et al., 2017; Uthman et al., 2019). Therefore, aPL are endothelial NO production antagonists, and NO reduction contributes to thrombi formation and leukocyte adhesion (Ramesh et al., 2011). However, the differences in aPL effect on different groups of patients, especially the refractory cases, are unclear.

Among the factors listed earlier, endothelial microparticles are 0.1–1 μm vesicles, presenting a procoagulant activity (Holnthoner et al., 2017). In cardiovascular disorders, an increase in the production of endothelial microparticles is detected by labeling with CD31 and annexin V, suggesting that these particles have the role as an endothelial dysfunction biomarker

Abbreviations: APS, antiphospholipid syndrome; aPL, antiphospholipid antibodies; β2GPI, beta 2-glycoprotein-I; IgG, immunoglobulin-G; VTI and VTII, primary and secondary thrombotic APS; RI+, refractory primary obstetric APS; NR+, non-refractory primary obstetric APS; RII+, refractory secondary obstetric APS; VTI/ aPL−, patients negative for aPL with thrombosis without autoimmune disease; VTII/ aPL−, patients negative for aPL with thrombosis and SLE; NHS, normal human serum; NO, nitric oxide; VEGF, vascular endothelial growth factor; LA, lupus anticoagulant; aCL, anti-cardiolipin antibodies; aβ2GPI, anti-β2-glycoprotein-I; HCQ, hydroxychloroquine; ROS, reactive oxygen species; VCAM-1, vascular cell adhesion molecule 1; NOS, nitric oxide synthase; MCP-1, monocyte chemoattractant protein I; FBS, fetal bovine serum; DCFH-DA, dichlorofluorescein diacetate; L-NAME, arginine methyl ester; DAF-FM-DA, 4-amino-5-methylamino-2′, 7-difluoroflurescein diacetate; ENX, enoxaparin; ASA, aspirin; 7-AAD, 7-aminoactinomycin D; HUVECs, human umbilical vein endothelial cells; MFI, median fluorescence intensity; FMO, fluorescence-minus-one.

(Deng et al., 2017; Leite et al., 2020). However, its difference in producing a different profile of microparticles in different groups of patients or its potential consequences on endothelial dysfunction is unclear.

Thus, the literature described aPL-mediated endothelial activation and dysfunction via an increase in the expression of adhesion molecules, oxidative stress, microparticle generation, and a decrease in NO, and little is known about the participation of β2GPI in these processes. Additionally, the different clinical characteristics of patients with APS in modifying aPL-mediated endothelial dysfunction are unclear. Therefore, this study aimed to evaluate the differences in endothelial activation and dysfunction induced by aPL between patients with refractory obstetric APS and other APS clinical manifestations. Moreover, the participation of β2GPI in aPL-mediated endothelial dysfunction was further explored. This knowledge would provide tools to evaluate new therapeutic strategies in refractory or more severe APS cases.

MATERIALS AND METHODS

Study Subjects

A total of 41 female patients were included in this study: 21 diagnosed with APS and 20 in the control group. According to clinical characteristics, female patients with obstetric APS and thrombosis (n = 11) were divided as follows: refractory primary (RI+, n = 3), refractory secondary [with systemic lupus erythematosus (SLE)] (RII+, n = 2), and non-refractory primary (NR+, n = 6). Refractoriness was defined as obstetric manifestations as patients manifest a new episode of pregnancy morbidity despite optimal pharmacological treatment with heparin and ASA during pregnancy (Mekinian et al., 2017). Patients with vascular thrombosis (n = 10) were classified as primary (VTI, n = 6) and secondary with SLE (VTII, n = 4). The control group included patients who were negative for aPL and with clinical manifestations of thrombosis (n = 10), classified as primary (patients who were negative for aPL with thrombosis without autoimmune disease (VTI/aPL-, n = 5) or secondary with SLE (patients who were negative for aPL with thrombosis and SLE (VTII/aPL-, n = 5) and patients with previous uncomplicated pregnancies [normal human serum (NHS), n = 10]. Exclusion criteria for all study participants were other associated diseases such as infections, diabetes, cancer, or chronic disease other than systemic autoimmune diseases due to the inclusion of patients with secondary APS. Patients were recruited from the Recurrent Pregnancy Loss Program of Reproduction Group (University of Antioquia) and Anticoagulation Clinic (San Vicente Fundación Hospital), with the previous approval of the Ethics Committee from the Medical Research Institute-School of Medicine (University of Antioquia). Informed consent was obtained from each participant. None of the patients were pregnant or presented an acute thrombosis episode when the samples were obtained.

Reagents, Materials, and Antibodies

Reagents, materials, and antibodies were acquired from the following manufacturers: Limulus amebocyte lysate, NuncTM filter flask, BODIPY C11, MitoSOX, eFluor, and anti-CD31-FITC from Thermo Scientific (Waltham, MA, United States); Type I collagenase from Invitrogen (San Diego, CA, United States); basal endothelial cell culture medium and fetal bovine serum (FBS) from Promocell (Heidelberg, Germany); RPMI-1640, FBS, Opti-MEM, and PBS from Gibco (Grand Island, NY, United States); penicillin and gentamicin from Genfar (Bogotá, Colombia); human-β2GPI from Louisville APL Diagnostics (Louisville, KY, United States); Amphotericin B, PKH67, dichlorofluorescein diacetate (DCFH-DA), propidium iodide, lipopolysaccharide (LPS), ASA, HCQ, vascular endothelial growth factor (VEGF) 165, and NG-nitro-L-arginine methyl ester (L-NAME) from Sigma (St. Louis, MO, United States); anti-VCAM-1-PE and anti-E-selectin-Alexa Fluor 700, from R&D Systems by Bio-techne (Minneapolis, MN, United States); 4-amino-5-methylamino-2′, 7-difluoroflurescein diacetate (DAF-FM-DA) from Cayman Chemical (Ann Arbor, MI, United States); enoxaparin (ENX) from Procaps (Barranquilla, Colombia); and annexin V and 7-aminoactinomycin D (7-AAD) from BD Pharmigen (San Diego, CA, United States).

Antiphospholipid Antibodies

The Clinical and Laboratory Standards Institute recommendations were followed for plasma LA determination (Pengo et al., 2009). APTT-SP (Instrumentation Laboratory, Orangeburg, NY, United States) was used to demonstrate the antibody dependence on phospholipids. Dilute Russell's viper venom time (dRVVT) screen and dRVVT confirm (Instrumentation Laboratory, Orangeburg, NY, United States) were used to detect LA. The presence of aCL and aβ2GPI was evaluated using commercial kits (BioSystems, Barcelona, Spain and Human, Wiesbaden, Germany, respectively) in serum and purified immunoglobulin-G (IgG). IgG from the serum pool of patients in each group was purified by affinity chromatography using protein G-Sepharose (General Electric Healthcare, NY, United States), as described (Alvarez et al., 2017). Endotoxins were detected in purified IgG using the Limulus amebocyte lysate assay.

Human Umbilical Vein Endothelial Cell Isolation

Human umbilical cords were obtained from female patients with uncomplicated pregnancies who attended obstetric services at the Hospital San Vicente Fundación, Medellín, Colombia. Voluntary female patients signed informed consent. The Ethical Committee of the same Hospital approved the sample collection. Human umbilical vein endothelial cells (HUVECs) were isolated by mechanical and enzymatic digestion based on the modified protocol of Jaffe (Jaffe et al., 1973; Gil-Villa et al., 2020). Briefly, the umbilical vein was perfused with a pericranial needle with 5 ml of type I collagenase followed by 20 min of incubation at 37°C. The umbilical vein content was centrifuged, and the button of cells (including HUVECs) was cultured in a NuncTM filter

flask with 10 ml of basal endothelial cell culture medium and 2% of FBS. Different umbilical cords from healthy patients were used to isolate HUVECs included in each experiment. HUVECs were used until passage three. In all the experiments, HUVECs were maintained in Opti-MEM in serum-free conditions to perform the stimuli with IgG and human-β2GPI.

THP-1 Cell Culture

THP-1 cells, derived from a 1-year-old infant with acute monocytic leukemia, were obtained from the American Type Culture Collection (CRL-1593, Manassas, VA, United States) and cultured in RPMI plus 10% FBS and antibiotic solution of 100 IU of penicillin, 50 μg of gentamicin, and 250 ng of amphotericin B at 37°C with 5% CO_2 and 60% relative humidity.

Evaluation of Endothelial Activation

Model of Adhesion

In 24-well plates, 5×10^4 HUVECs were added per well to evaluate the expression of E-selectin and vascular cell adhesion molecule 1 (VCAM-1) induced by 50 μg/ml IgG for 24 h with or without 5 μg/ml human-β2GPI. Subsequently, the cells were detached by incubating with trypsin for 2 min, deactivated with 2% FBS, and washed with PBS. The following antibodies were added (diluted 1:100): anti-VCAM-1-PE and anti-E-selectin-Alexa Fluor 700. Non-specific bindings were blocked with 20% FBS in PBS. The cells were evaluated using a flow cytometer LSR Fortessa (BD), acquiring 10,000 events per sample. The obtained data were analyzed using the FlowJo® v7.6.2 software. Positive cell values are indicated as the percentage and median fluorescence intensity (MFI). The fluorescence-minus-one (FMO) control included all antibodies with the conjugated fluorochromes except the molecules of interest. The FMO control was prepared for each antibody. Subsequently, the effect of IgG on the monocyte adhesion to the endothelium was detected. THP-1 cells were labeled with the fluorescent dye PKH67 according to the manufacturer's instructions. Briefly, in 24-well plates, 0.2 μl of PKH67 and 20 μl of diluent C (included with the kit) were added to each 1×10^4 cells per well. After that, 80 μl of FBS was added to remove excess dye, and the cells were incubated with 250 μg/ml of IgG for 24 h. Simultaneously, 1×10^4 HUVECs per well were stimulated with IgG under the same conditions like THP-1. After 24 h of IgG stimulation, THP-1 monocytes were added to the HUVEC monolayer and incubated for 2 h. Finally, the cells were washed with PBS at 37°C to remove non-adherent cells. THP-1 cells attached to the PKH67-labeled endothelium were detected using the spectrofluorometer Varioskan TM LUX multimode microplate reader (Thermo Scientific, Waltham, MA, United States). Photographs were acquired using the DS-Fi1 camera (Nikon, Shinagawa, Japan) adapted to the Axio Vert.A1 fluorescence microscope (Zeiss, Berlín, Germany) with a 20X objective. LPS (4 μg/ml) was included as a positive control for E-selectin and VCAM-1 expression and the THP-1 cell adhesion to the endothelium. All stimuli were performed in Opti-MEM plus antibiotics at 37°C under 5% CO_2 and 60% relative humidity.

Oxidative Stress Evaluation

Oxidative stress was evaluated as an indicator of endothelial activation by the intracellular ROS, superoxide anion (O_2^-) production, and lipid peroxidation in aPL-stimulated endothelial cells. In 24-well plates, 5×10^4 HUVECs were added per well and stimulated for 24 h with 250 μg/ml IgG. The cells were detached using trypsin, which was inactivated with Opti-MEM containing 10% FBS. The cells were washed two times by centrifugation at $580 \times g$ for 5 min with 600 μl of PBS. To detect ROS production, 0.05 μM of DCFH-DA and 0.5 μM of propidium iodide were added to the cells. Then, 0.825 μM of BODIPY C11 in 1,200 μl of PBS was added to detect membrane lipid peroxidation. For the evaluation of mitochondrial O_2^- production evaluation, 0.02 μM MitoSOX probe with 0.01 μM eFluor was added to the cells. Using the LSR Fortessa flow cytometer (BD), 1×10^4 events per sample were acquired. The obtained data were analyzed using the FlowJo® v7.6.2 program. Values were reported as MFI. Cells were stimulated with 0.5 and 1 mM hydrogen peroxide as an endothelial oxidative stress positive control. About 2, 4, and 8 μg/ml of LPS were used as a positive control to induce O_2^-. The cells were incubated with 5 μg/ml of β2GPI to detect the effect of aβ2GPI in IgG treatment.

Detection of Endothelial Dysfunction

Generation of Cell-Derived Endothelial Microparticles *in vitro*

The generation of cell-derived endothelial microparticles *in vitro* was detected based on the Pericleous protocol (Pericleous et al., 2013). Briefly, HUVECs were treated with IgG or 8 μg/ml of LPS for 24 h, and the supernatants were collected and centrifuged at $3{,}000 \times g$ for 5 min to remove debris. The supernatant was centrifuged at $15{,}000 \times g$ for 60 min to obtain the cell-derived endothelial microparticles, which were resuspended in a filtered binding buffer and stained with 1:100 of anti-CD31 and annexin V. Readings were made on a flow cytometer, acquiring total events in 120 s. Cell-derived microparticle size was defined using 0. 5-, 1-, and 2-μm polystyrene beads. The levels of basal and LPS-induced cell-derived endothelial microparticles and the signals from polystyrene beads were differentiated from the electronic noise. The data were analyzed using the FlowJo® v7.6.2 program. A number of events were reported as microparticles.

Nitric Oxide Release

Nitric oxide bioavailability was evaluated using the probe DAF-FM-DA in the HUVECs stimulated with aPL. DAF-FM-DA is a cell-permeable probe; once NO crosses the plasma membrane, this dye is deacetylated by intracellular esterases and is transformed into DAF-FM. DAF-FM has a baseline fluorescence of 0.005 but increases to 0.81 (160 times) when it reacts with NO with an excitation/emission maximum of 495/515 nm. In 24-well plates, 5×10^4 HUVECs were added per well and stimulated for 1 h with 250 μg/ml of IgG with or without 5 μg/ml of β2GPI. After incubation with IgG, HUVECs were washed with PBS, and 1 μM of DAF-FM-DA was added, followed by 20 min of incubation at 37°C. Fluorescence of DAF-FM-DA was analyzed using the Varioskan TM LUX multimode microplate reader (Thermo Scientific, Waltham, MA,

United States). As positive controls to induce NO release, 100 and 200 ng/ml of VEGF165 were included. As a negative control for NO production, 100 μM of the nitric oxide synthase (NOS) antagonist, L-NAME, was used.

Endothelin-1 and Vascular Endothelial Growth Factor Production

This study detected endothelin-1 and VEGF production in supernatants of HUVECs treated (24 h) with IgG with or without β2GPI. Both endothelin-1 (R&D) and VEGF (Invitrogen, Waltham, MA, United States) were measured using human ELISA kits according to the respective manufacturer's instructions.

Modulation of Endothelial Dysfunction

Aspirin at 10 mM, ENX at 50 IU/ml, and HCQ at 1 μg/ml were simultaneously added with IgG to detect the modulating effect on NO release.

Assessment of Cell Viability

The effect of IgG, ASA, ENX, HCQ, and β2GPI on cell viability was evaluated using 7-AAD. The stimuli were performed under the same conditions previously described to detect NO. Following 1-h incubation, the dead cells of 7-AAD+ were detected using an LSR Fortessa flow cytometer (BD). The obtained data were analyzed using the FlowJo® v7.6.2 program and reported as% dead cells. As a positive control of dead cells, 4- and 8-mM hydrogen peroxide (Sigma, St. Louis, MO, United States) were used.

Statistical Analysis

Data distribution was evaluated using the Shapiro–Wilk tests. Data are expressed as mean ± SD. A *t*-test, Mann–Whitney test and two-way ANOVAII were performed, and the comparison between means was determined using the Holm–Sidak post-test. Statistical analyses were performed using Prism6 (GraphPad Software, Inc., San Diego, CA, United States). For all the cases, the value of $p < 0.05$ was considered significant.

RESULTS

Patient Characteristics

No differences were observed in the age of patients of different groups. The samples from patients with VTI, RI+, RII+, and NR+ were positive for aPL in the serum and purified IgG. Contrarily, the group with VTII alone was positive for LA and serum aCL. The RII+ group was positive for aPL; however, it had lower values than patients with refractory and NR+ APS (RI+ and NR+). Patients with RI+ had higher LA values compared with other groups. Two patients in this group had catastrophic APS. The IgG samples of controls (NHS, VTI/aPL-, and VTII/aPL-) were negative for aPL (**Table 1**). All the tested IgG samples were negative for endotoxins. For all the cases, secondary APS indicated the presence of SLE. All patients with refractory APS had pregnancy morbidity even after ASA and heparin treatment (**Table 1**). Different characteristics of thrombotic events were observed among patients with thrombosis from which the received treatment was derived (**Table 1**).

Immunoglobulin-G From Patients With Primary Thrombotic Antiphospholipid Syndrome and RI+ Induced an Increase in Expression of Adhesion Molecule as an Endothelial Activation Indicator

In all the experiments, LPS increased E-selectin and VCAM-1 expressions (**Figures 1A,E**). In β2GPI-stimulated endothelial cells, the VTI and RI+ group IgG increased E-selectin compared with NHS control IgG (**Figures 1B–D**). Also, in β2GPI-stimulated endothelial cells, RI+ IgG increased VCAM-1 in contrast with the NHS control IgG (**Figures 1F,G**).

Immunoglobulin-G From Patients With RI+ Induced an Increased Monocyte Adhesion

Monocyte aggregates were not observed in the endothelium regarding basal adhesion and cells treated with VTI/aPL, VTII/aPL, and NHS controls (**Figures 2A,C–E**). As expected, LPS increased the adhesion of THP-1 monocytes to an endothelial monolayer (**Figures 2B,G**). Complementarily, RI+ IgG increased the adhesion of monocytes to the endothelium compared with VTI/aPL and VTII/aPL controls (**Figures 2D–F,H**). The number of aggregates was higher and statistically significant upon RI+ stimulation (**Figures 2F,H**).

Immunoglobulin-G From Patients With RII+ Induced an Augmentation in O_2^- Production

Hydrogen peroxide and LPS increased the endothelial O_2^- production (**Figures 3A,B,D**). RII+ IgG increased endothelial O_2^- production compared with the NHS and NR+ IgG without β2GPI (**Figures 3C,E**). No IgG effect was observed in cells treated with β2GPI (**Figure 3E**). In addition, IgG from the patient groups included in this study and β2GPI alone did not affect the ROS production detected by DCF signal or lipoperoxidation by BODIPY C11 staining (**Supplementary Figure 1**). IgG from patients with positive or negative aPL and thrombosis (VTI/aPL-, VTII/aPL-, VTI, and VTII) did not affect the generation of oxidative stress in HUVECs (data not shown).

Immunoglobulin-G From Patients With NR+ and RII+ Increased the Cell-Derived Endothelial Microparticles

A higher number of 0.5- and 1-μm endothelial microparticles for CD31+/annexin V-, CD31+/annexin V+, CD31-/annexin V+, and CD31-/annexin *V*- were generated in cells stimulated with LPS compared with basal microparticle production (**Supplementary Figures 2A–N**). After the initial set up of an experimental protocol, including a potential experimental confounder generated by electronic noise or negligible count of particles in the binding buffer (**Figures 4A,B**) and using LPS

TABLE 1 | Characteristics of patients included in this study.

Characteristics	NHS ($n = 10$)	VTI/aPL- ($n = 5$)	VTII/aPL- ($n = 5$)	VTI ($n = 6$)	VTII ($n = 4$)	RI+ ($n = 3$)	RII+ ($n = 2$)	NR+ ($n = 6$)
Age (mean of years ± SD)	35.5 ± 5.46	38.8 ± 15.61	42.8 ± 12.09	30 ± 11.21	35.25 ± 11.95	34.33 ± 6.81	46.5 ± 3.54	38.83 ± 7.22
Pregnancy loss (mean and rank) ≤10 weeks of pregnancy	0	0	0	0	0	2.33 (2-5)	1 (1-2)	1.2 (1-5)
>10 weeks of pregnancy	0	0	0	0	0	2.33 (1-5)	3 (1-5)	0.6 (0-2)
Preeclampsia < 34 weeks (number of patients)	0	0	0	0	0	3	1	2
Intrauterine growth restriction (number of patients)	0	1	0	0	0	2	0	1
Arterial thrombosis (number of patients)	0	0	0	1	0	0	0	0
Deep vein thrombosis (number of patients)	0	3	2	3	4	3	2	6
Pulmonary embolism (number of patients)	0	1	5	3	2	1	1	3
Stroke (number of patients)	0	2	0	1	1	0	1	0
Recurrent thrombosis (number of patients)	0	3	3	2	4	3	2	3
Warfarin (number of patients)	0	3	3	6	4	1	2	3
Prednisolone (number of patients)	0	3	2	0	2	0	1	0
Statins (number of patients)	0	0	0	0	0	0	1	0
Chloroquine (number of patients)	0	2	1	1	1	1	2	2
Hydroxychloroquine (number of patients)	0	0	0	0	1	0	0	0
Lupus anticoagulant (mean ± SD)[†]	1.06 ± 0.05	1.13 ± 0.01	1.10 ± 0.02	2.62 ± 0.16 (+)	2.26 ± 0.6 (+)	3.3 ± 0.24 (+)	1.34 ± 0.24 (+)	1.92 ± 0.27 (+)
IgG anti-β2 glycoprotein I (serum) (U/ml) (mean ± SD)[‡]	2.29 ± 0.07	2.52 ± 0.32	2.08 ± 0.33	56.37 ± 8.79 (+)	5.9 ± 0.44	97.88 ± 14.02 (+)	16.56 ± 5.1 (+)	67.79 ± 32.5 (+)
IgG anti-cardiolipin (serum) (GPL/ml) (mean ± SD)[£]	0	1.08 ± 0.15	0	97.09 ± 6.96 (+)	12.8 ± 0.51 (+)	102.2 ± 15.58 (+)	29.71 ± 1.1 (+)	93.73 ± 2.56 (+)
IgG anti-β2 glycoprotein (mean ± SD)[‡]	0.84 ± 0.01	0.59 ± 0.08	0.72 ± 0.38	14.09 ± 21.54 (+)	3.95 ± 3.9	21.78 ± 25.96 (+)	7.9 ± 6.6 (+)	38.55 ± 32.43 (+)
IgG anti-cardiolipin (mean ± SD)[£]	0	0	0	10.34 ± 1.95 (+)	3.93 ± 0.87	34.7 ± 1.82 (+)	16.54 ± 0.5 (+)	80.03 ± 1.63 (+)

GPL [immunoglobulin-G (IgG) phospholipid units]. IgG anti-β2-glycoprotein and IgG anti-cardiolipin were evaluated in 250 μg/ml of purified IgG.

[†]*Values greater than 1.2 indicate a positive test for lupus anticoagulant (LA).*

[‡]*Values greater than 7 U/ml anti-β2-glycoprotein-I are positive.*

[£]*Values greater than 10 GPL/ml anti-cardiolipin are positive.*

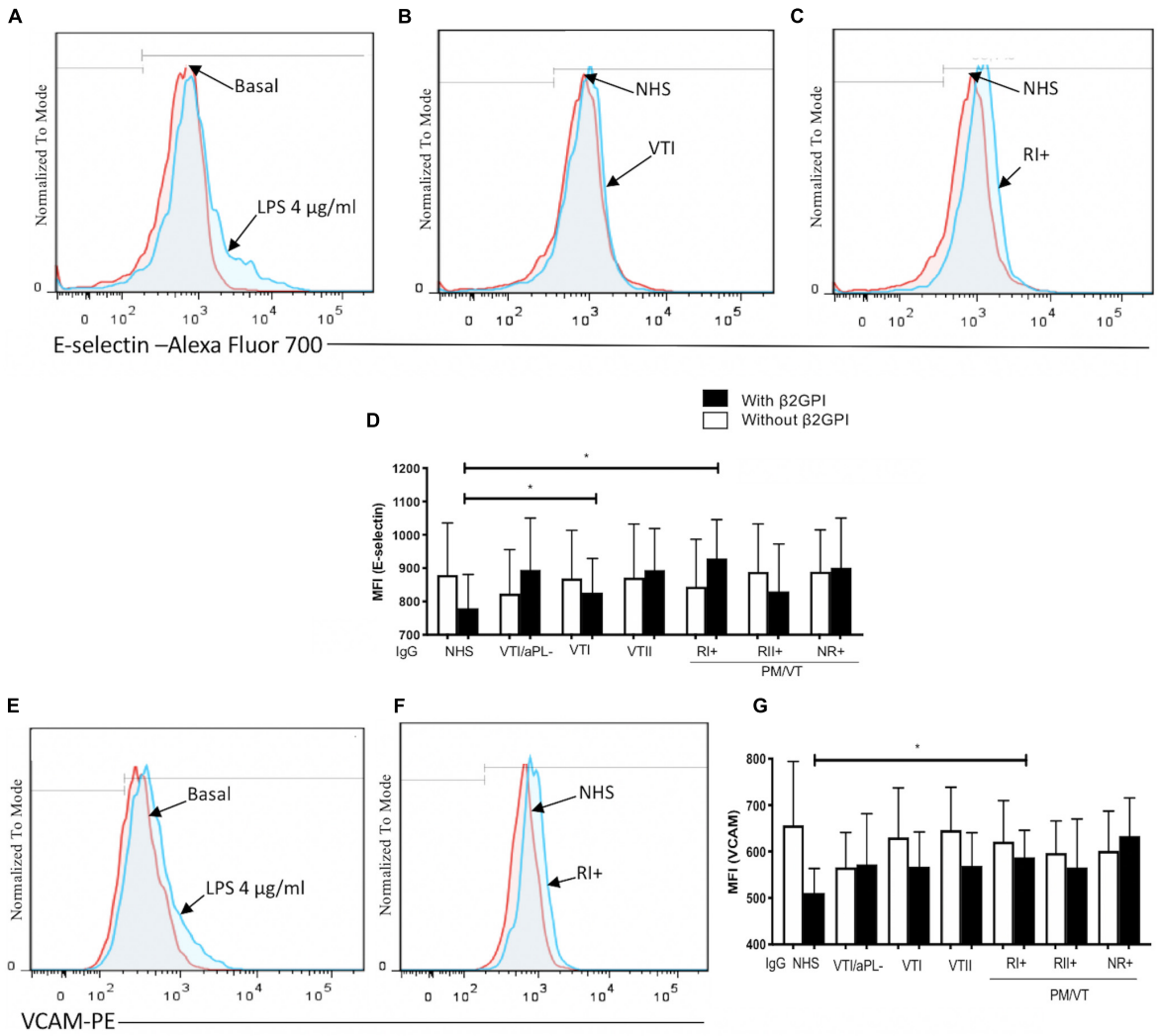

FIGURE 1 | Immunoglobulin-G (IgG) from patients with primary antiphospholipid syndrome (APS) [primary thrombotic APS (VTI) and refractory primary obstetric APS (RI+)] induced an increase in the adhesion molecule expression in human umbilical vein endothelial cells (HUVECs). **(A,E)** Lipopolysaccharide (LPS) at a concentration of 4 µg/ml was included as a positive control, and the increased E-selectin and vascular cell adhesion molecule 1 (VCAM-1) expression was compared with unstimulated cells. **(B–D)** The median fluorescence intensity (MFI) of E-selectin increased in HUVECs stimulated with beta 2-glycoprotein-I (β2GPI) and IgG from patients with VTI and RI+ compared with the IgG/normal human serum (NHS) control. **(F,G)** The MFI of VCAM-1 increased in HUVECs stimulated with β2GPI and IgG from patients with RI+ compared with the IgG/NHS control. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$ and **$p < 0.01$) were performed. The results were obtained from three independent experiments. **(A–C,E,G)** Representative histograms. PM/VT, pregnancy morbidity and vascular thrombosis.

as a positive control to increase the total microparticle count (**Figures 4C,D**), the effect of IgG from patients with APS was tested. In addition, the microparticle size was estimated using the polystyrene beads of 0.5, 1, and 2 µm (**Figure 4E**). Contrarily, NR+ IgG with β2GPI increased the generation of 0.5-µm CD31+/annexin V+ microparticles compared with IgG from the same group without β2GPI and NHS IgG, VTII/aPL-, and VTII controls with β2GPI (**Figures 5B–F,K**). IgG from this group with β2GPI increased the generation of 1-µm CD31+/annexin *V*- microparticles compared with IgG without β2GPI or NHS control IgG (**Figures 5H–J,L**). RII+ IgG increased the production of 1-µm endothelial dysfunction biomarkers of the total cell-derived endothelial microparticles. RII+ IgG increased the generation of microparticles (1 µm) in a β2GPI-dependent manner compared with the NHS control (**Figures 4F–H**).

Immunoglobulin-G From Patients With NR+ and Primary Thrombotic Antiphospholipid Syndrome Induced Production of Procoagulant (Annexin V+) Cell-Derived Endothelial Microparticles

Non-refractory primary obstetric APS and VTI IgG increased the procoagulant microparticles or Annexin V+ (CD31+/annexin V+ and CD31-/annexin V+) by 0.5 µm (**Figure 5M**). IgG from

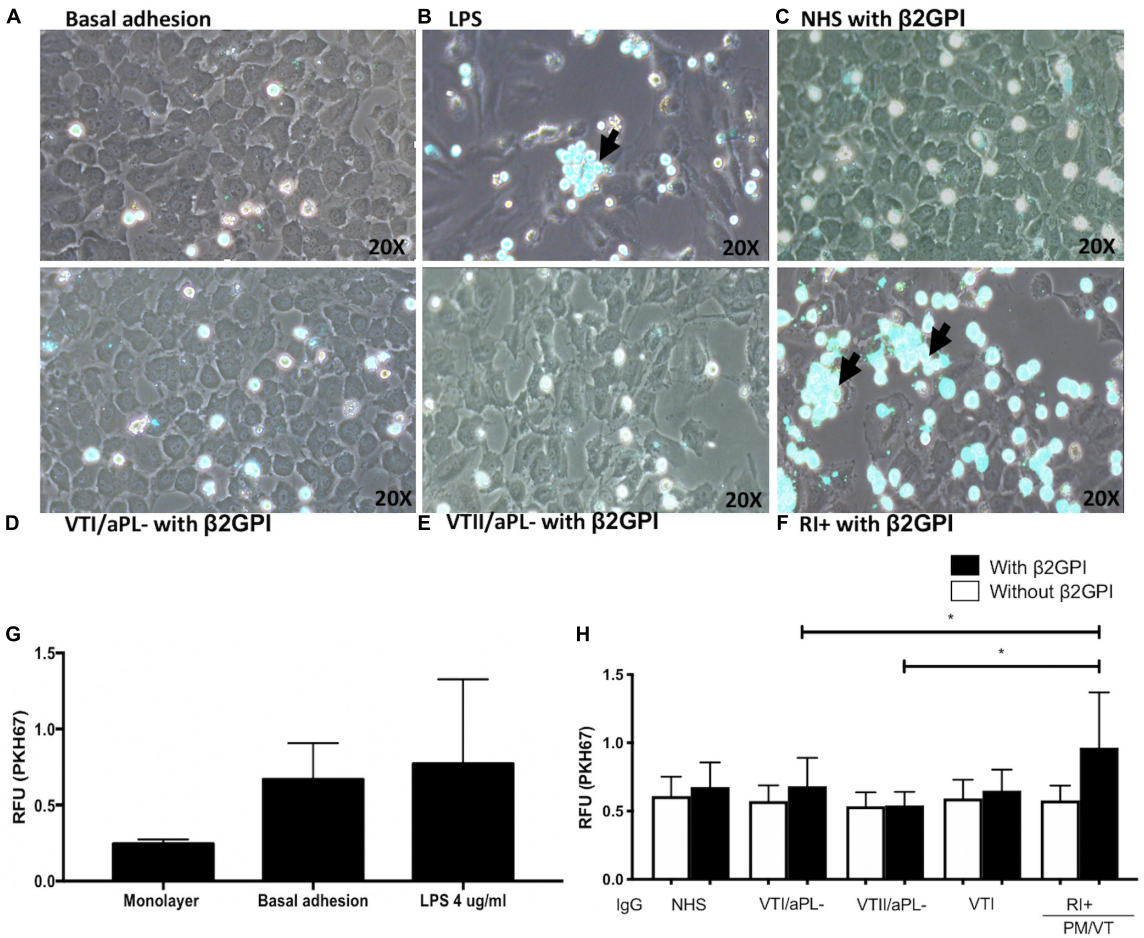

FIGURE 2 | Immunoglobulin-G from patients with refractory and primary obstetric APS (RI+) induces increased monocyte adhesion to the endothelium. **(A)** Baseline adhesion. **(B)** LPS. The adhesion of monocytes to the endothelium in cells stimulated with β2GPI plus: **(C)** IgG/NHS; **(D)** IgG/patients negative for aPL with thrombosis without autoimmune disease (VTI/aPL-); **(E)** IgG/patients negative for aPL with thrombosis and SLE (VTII/aPL-); and **(F)** IgG/RI+. **(A–F)** Representative images. **(G)** The results obtained using the spectrofluorometer are indicated in relative fluorescence units (RFU) emitted by the endothelial monolayer without monocytes and with monocytes attached without stimulus or baseline adhesion and with LPS. **(H)** IgG from patients with RI + and β2GPI increased the adhesion of monocytes compared with IgG from patients with VTI/aPL- and VTII/aPL-. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$) were performed. The results were obtained from three independent experiments. The arrow indicates the aggregates of monocytes. PM/VT, pregnancy morbidity and vascular thrombosis.

the patient groups included in this study did not induce 2-μm microparticles (data not shown). The dot-plot labeled with control indicates microparticles without antibodies, which were used to define the location of negative and positive MPs for Annexin V and CD31 (**Figures 5A,G**).

All Immunoglobulin-G From Patients With Pregnancy Morbidity and Thrombosis (RI+, RII+, and NR+) Induced Endothelial Dysfunction Through a Decreased Nitric Oxide Production in Human Umbilical Vein Endothelial Cells

Vascular endothelial growth factor-induced NO synthesis in a dose-dependent manner, whereas L-NAME inhibited its production in all the VEGF-used doses (**Figures 6A,B**). β2GPI alone did not alter NO synthesis in contrast with the baseline control (**Figure 6A**). RI+ IgG decreased NO synthesis in HUVECs compared with NHS control IgG without β2GPI (**Figure 6C**). All IgG from patients with pregnancy morbidity and thrombosis (RI+, RII+, and NR+) reduced NO synthesis compared with the NHS IgG control in a β2GPI-dependent manner (**Figure 6C**). ASA did not induce a modulatory effect on reducing NO induced by IgG from patients with pregnancy morbidity and thrombosis (**Figures 6D,E**). ENX restored the reduced synthesis of NO generated by RI+ IgG with and without β2GPI (**Figures 6F,G**). HCQ did not induce a modulatory effect on reducing NO induced by IgG from patients with pregnancy morbidity and thrombosis without β2GPI (**Figure 6I**). HCQ restored the synthesis of NO reduced by IgG from RI+ with β2GPI (**Figure 6H**). IgG, ASA, EXN, and HCQ

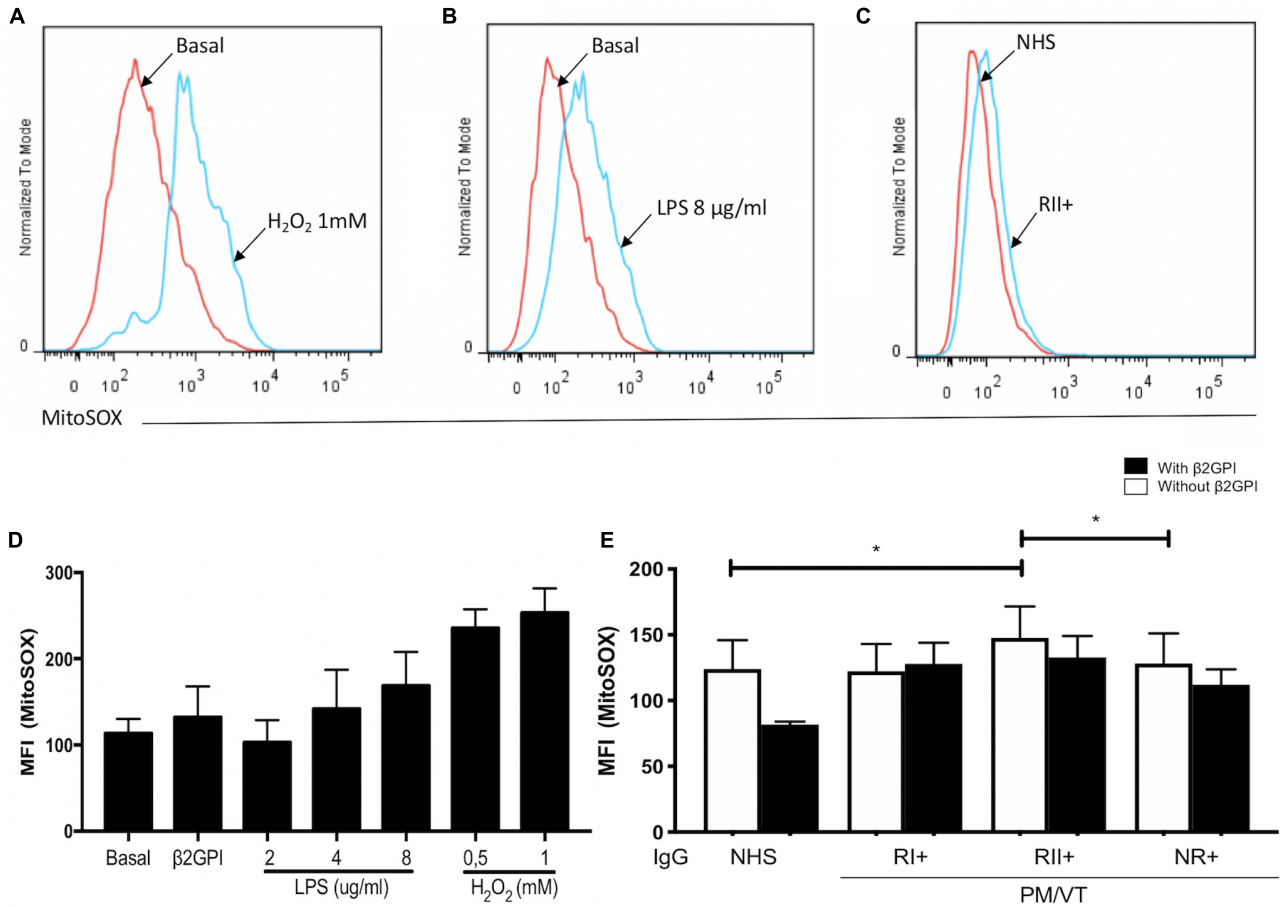

FIGURE 3 | Immunoglobulin-G from patients with refractory and secondary obstetric APS (RII+) induced O_2^- mitochondrial production. **(A,B,D)** Hydrogen peroxide and LPS increased the MFI of MitoSOX in HUVECs. **(C,E)** RII + IgG group induced mitochondrial O_2^- production in HUVECs without β2GPI compared with the IgG from NHS and non-refractory primary obstetric APS (NR+) groups. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$) were performed. The results were obtained from five independent experiments. **(A–C)** Representative histograms. PM/VT, pregnancy morbidity and vascular thrombosis.

did not induce dead cells compared with baseline control (**Supplementary Figure 3**).

All Immunoglobulin-G From Patients With Antiphospholipid Syndrome Induced Endothelial Dysfunction Through an Alteration in Endothelin-1 or Vascular Endothelial Growth Factor Production in Human Umbilical Vein Endothelial Cells Supernatants

Refractory secondary obstetric APS and NR+ IgG increased the presence of endothelin-1 compared with NHS IgG without the addition of β2GPI (**Figure 7A**). RI+ IgG with β2GPI increased the production of endothelin-1 compared with RI+ IgG without β2GPI (**Figure 7A**). IgG from patients with VTI and VTII increased the VEGF in contrast with NHS IgG in the absence of β2GPI. IgG from patients with NR+ decreased the VEGF levels in contrast with IgG from the NHS in the absence of β2GPI. NR+ IgG with β2GPI increased VEGF levels compared with that without β2GPI (**Figure 7B**).

DISCUSSION

Endothelial Activation and Dysfunction in Antiphospholipid Syndrome: Association With Clinical Manifestations

Different endothelial activation and dysfunction mechanisms induced by IgG in groups of patients included in our study are associated with the development of APS clinical manifestations. Our main findings include: (1) VTI IgG increased the expression of E-selectin, generated positive procoagulant microparticles, and increased VEGF generation (**Figure 8A**). (2) RI+ IgG increased the adhesion of monocytes and decreased NO, which were modulated by HCQ and ENX (**Figure 8B**). The results suggest that HCQ and ENX together become an effective alternative for these patients, considering the endothelial

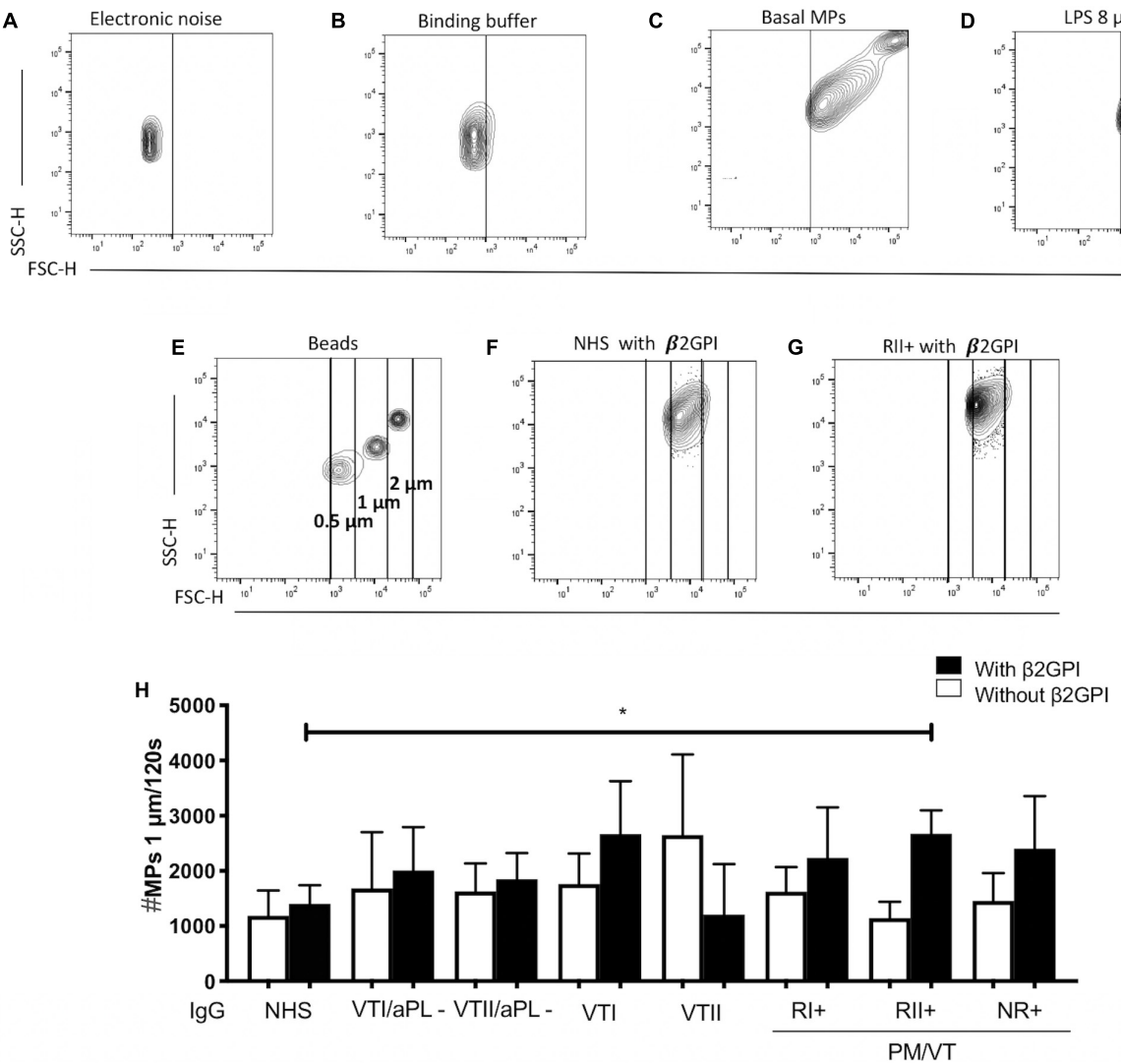

FIGURE 4 | Immunoglobulin-G from patients with secondary refractory APS (RII+) increased the number of 1-µm microparticles (MPs). **(A)** Electronic noise was excluded from all the analyses. **(B)** The binding buffer for annexin V staining did not present a significant number of events. **(C,D)** LPS stimulation increased the number of MPs compared with no stimulation. **(E)** For all the analyses, the approximate size of the MPs was delimited with 0. 5-, 1-, and 2-µm beads. **(F–H)** IgG of patients with RII + induced the generation of 1-µm MPs in a β2GPI-dependent manner compared with IgG from NHS control. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$) were performed. The results were obtained from three independent experiments. PM/VT, pregnancy morbidity and vascular thrombosis.

dysfunction modulation induced by the previous activation of these cells. (3) NR+ IgG decreased NO bioavailability, increased procoagulant microparticles, induced endothelin-1, and reduced VEGF production (**Figure 8C**). In these NR+ patients, heparin and ASA could modulate the clinical manifestations using the mechanisms that were not evaluated in this study. (4) IgG from RII+ decreased NO bioavailability, generated the production of microparticle and endothelin-1, and induced oxidative stress (mitochondrial O_2^- production) (**Figure 8D**). In these groups of patients, the antioxidant therapy, which was proposed by a few authors, was used for APS treatment.

Both the aPL that require or do not require β2GPI to increase their pathological effect on the endothelium could act synergistically in each group of patient to induce clinical manifestations. aPL are associated with different clinical manifestations of APS, but the mechanism that explains these associations or the presence of these autoantibodies or epitopes in distinct groups of patients with APS is unknown, especially in the refractory cases. aPL generate endothelial activation leading to cell dysfunction and favor clinical manifestations of thrombosis and gestational morbidity. These aPL recognize antigens, such as cardiolipin and β2GPI, and epitopes of these antigens that determine the pathological effect or clinical manifestation. An example of this is the antibodies anti-domain-I of β2GPI (aD1-β2GPI) in the glycine40-arginine 43 associated with thrombosis (de Laat et al., 2005). However, the presence of these aD1-β2GPI

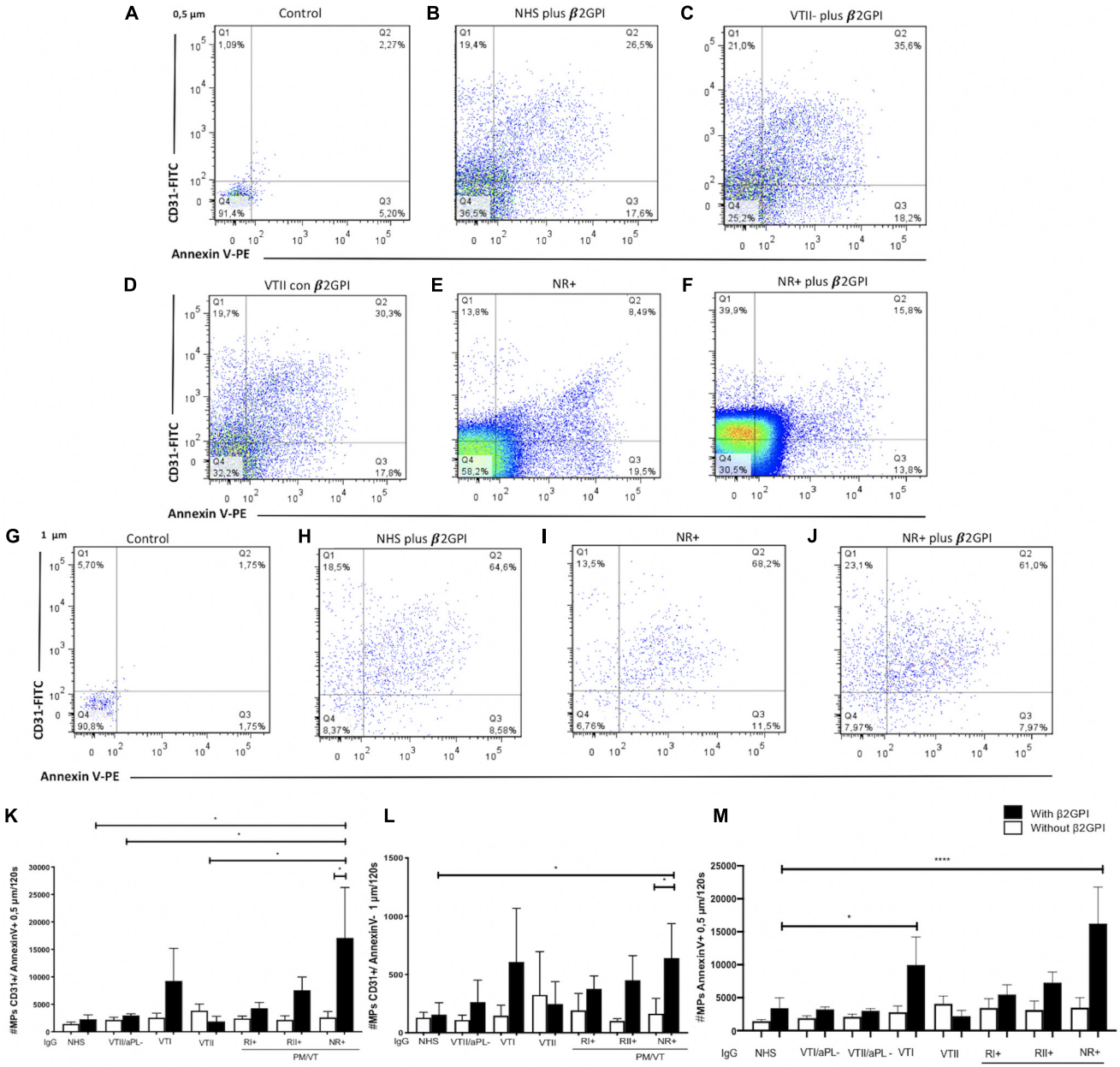

FIGURE 5 | Immunoglobulin-G from patients with non-refractory primary obstetric APS (NR+) induced the production of 0.5- and 1-μm endothelial MPs. **(A,G)** Control indicates MPs without antibodies, which were used to define the location of the negative and positive MPs for Annexin V and CD31. **(A–F,K)** IgG/NR+ increased the number of 0.5-μm CD31+/annexin V+ MPs in the presence of β2GPI, compared with NHS IgG, secondary thrombotic APS (VTII)/antiphospholipid antibodies (aPL) IgG, VTII IgG, and IgG of patients with NR+ without β2GPI. **(G–J,L)** IgG from patients with NR+ increased the number of MPs of 1-μm CD31+/annexin V- in a β2GPI-dependent manner compared with the IgG/NHS control and patients with NR+ without β2GPI. **(M)** IgG from patients with VTI and NR+ increased the number of total MPs of 0.5-(μm annexin V (in a (β2GPI-dependent manner more than IgG/NHS; Annexin V (MPs are the indicators of procoagulant potential. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$) were performed. The results were obtained from three independent experiments. PM/VT, pregnancy morbidity and vascular thrombosis. ****$p < 0.0001$.

is a predictor of thrombosis and pregnancy morbidity; contrarily, the antibodies anti-domain-4/5 of β2GPI is not associated with these clinical manifestations (Chighizola et al., 2018). Additionally, patients with SLE, LA, and aβ2GPI IgA antibodies are also associated with developing thrombosis and aCL with preterm delivery (Saleh et al., 2020; Demir et al., 2021). Patients with different clinical manifestations, refractoriness, and other autoimmune diseases, such as SLE, were included to differentiate between the pathological mechanisms of aPL on endothelial activation and dysfunction that explain the aPL generation of

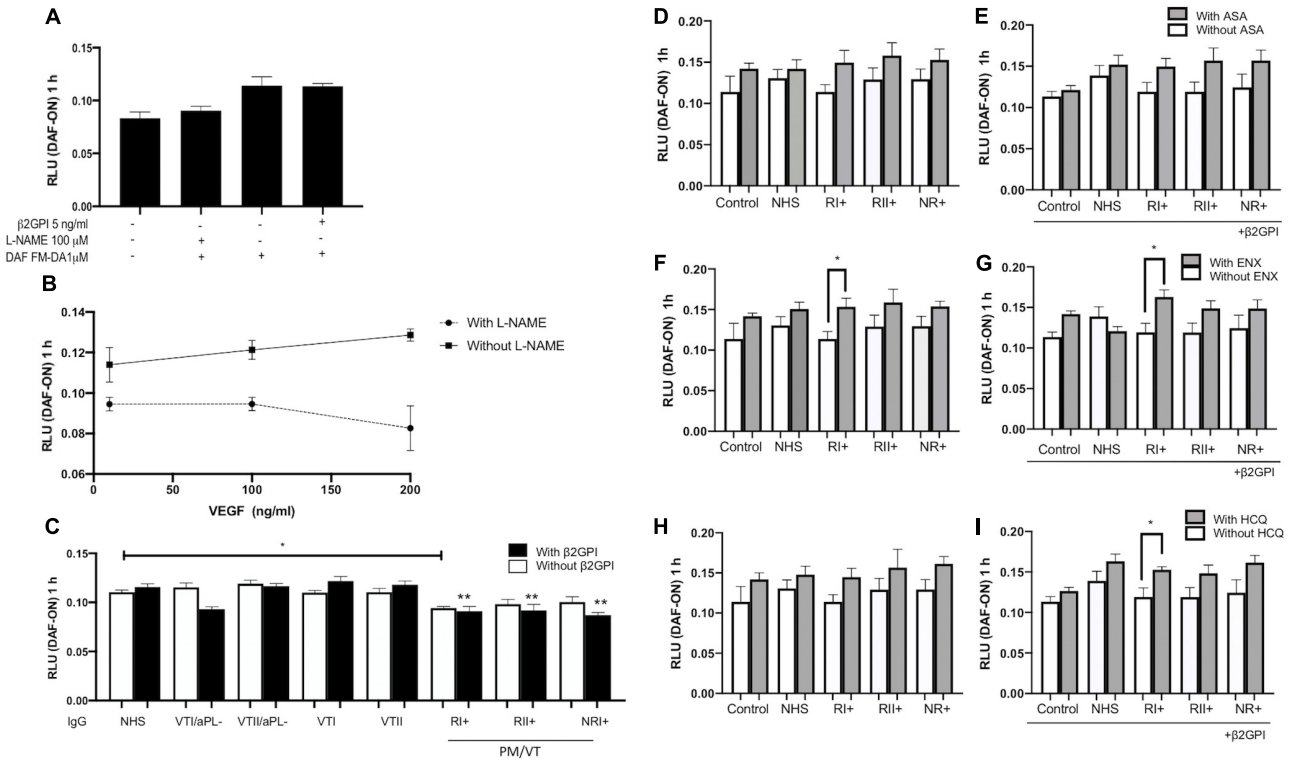

FIGURE 6 | All IgG from patients with pregnancy morbidity and thrombosis (RI+, RII+, and NR+) induced endothelial dysfunction through a decrease in nitric oxide (NO) production in HUVECs. **(A)** β2GPI alone did not alter NO production in contrast with the basal control. **(A,B)** NO release is reduced by the addition of NG-nitro-L-arginine methyl ester (L-NAME). **(B)** Vascular endothelial growth factor (VEGF) induced NO production in a dose-dependent manner in contrast with cells treated with L-NAME, which exerted its antagonistic effect. **(C)** IgG/RI+ decreased NO bioavailability in contrast with the IgG/NHS control without β2GPI. All IgG from patients with pregnancy morbidity and thrombosis (RI+, RII+, and NR+) plus β2GPI reduced NO production compared with the IgG/NHS control (**$p < 0.01$). A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$) were performed. **(D,E)** Aspirin (ASA) had no modulatory effect on the reduction of NO induced by IgG. **(F,G)** Enoxaparin (ENX) restored NO bioavailability reduced by IgG/RI+ with and without β2GPI. **(H,I)** Hydroxychloroquine (HCQ) restored NO bioavailability reduced by IgG/RI+ with β2GPI. A *t*-test (*$p < 0.05$) was performed in ASA and EXN analyses. The HCQ data were analyzed using the Mann–Whitney test (*$p < 0.05$). The results were obtained from four independent experiments. PM/VT, pregnancy morbidity and vascular thrombosis.

different pathological effects and triggering thrombosis alone or thrombosis with pregnancy morbidity.

Proadhesive Phenotype as an Indicator of Endothelial Activation Leading to Endothelial Dysfunction

Immunoglobulin-G from the different groups of patients triggered different mechanisms to generate endothelial activation and dysfunction that explain APS clinical manifestations. RI+ IgG with β2GPI induced endothelial activation by expressing E-selectin, VCAM, and consequent monocyte adhesion to the endothelium. Both molecules increased by aPL indicate endothelial activation associated with thrombus formation (Gandhi et al., 2021). In our experimental model, VTI IgG did not induce endothelial dysfunction as evaluated with NO synthesis. RI+ IgG induced endothelial activation and dysfunction. RI+ IgG induced endothelial dysfunction due to the NO bioavailability reduction with and without β2GPI. The expression of adhesion molecules is mediated by the factor NF-kB that is inhibited by NO (Liao, 2013). NO produced by eNOS or NO-donors reduces endothelial activation by inhibiting the expression of adhesion molecules, leukocyte adhesion and traffic, platelet reactivity, and vascular proliferation and angiogenesis modulation (Liao, 2013). The expression of adhesion molecules allowing the binding of monocytes to the endothelium and the decreased NO were associated with thrombosis (Ghimire et al., 2017). Monocytes are the primary sources of tissue factor, a key in the extrinsic coagulation cascade. These cells also bind platelets to each other and participate in thrombus recanalization (Mukhopadhyay et al., 2019). On the other hand, NO reduction represents a prothrombotic microenvironment considering that NO inhibits platelet and leukocyte adhesion to the endothelium (Costa et al., 2019). These mechanisms of endothelial activation and dysfunction induced by RI+ IgG associated with thrombosis also lead to gestational morbidity (Possomato-Vieira and Khalil, 2016). Therefore, decreased NO was associated with increased vasoconstriction and hypertension, leading to an alteration in adequate uterine spiral artery remodeling (Possomato-Vieira and Khalil, 2016). In addition, similar monocyte-mediated activation of endothelial cells was described in preeclampsia, a pregnancy condition characterized

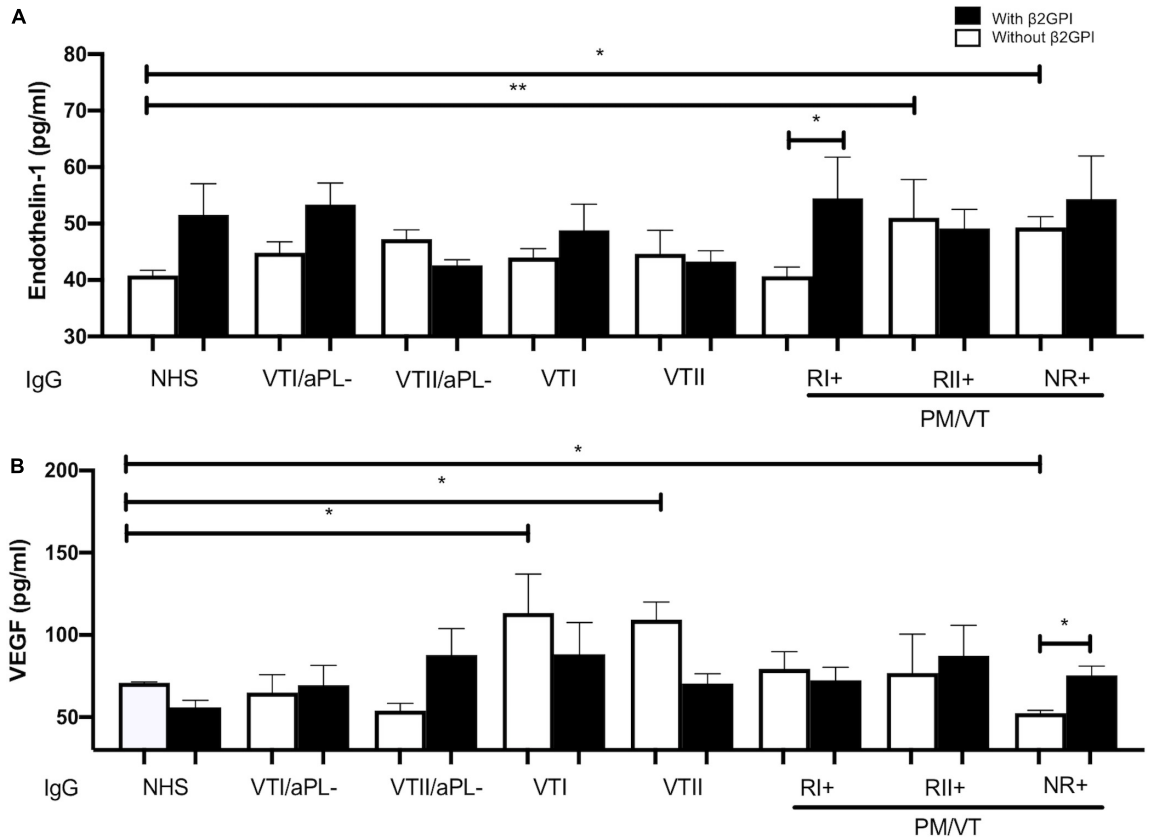

FIGURE 7 | All IgG from patients with APS-induced endothelial dysfunction through an alteration in endothelin-1 or VEGF production in HUVEC supernatants. **(A)** RII+ and NRI+ IgG groups of patients increased endothelin-1 production compared with IgG/NHS without β2GPI. RI+ IgG with β2GPI increased the production of endothelin-1 compared with this same group without β2GPI. **(B)** IgG from patients with VTI and VTII increased the VEGF in contrast with NHS IgG without β2GPI. IgG from patients with NR+ decreased the VEGF amount in contrast with NHS IgG without β2GPI. NR+ IgG and β2GPI increased the VEGF production compared with this same IgG without β2GPI. A two-way ANOVA and the Holm–Sidak post-test (*$p < 0.05$; **$p < 0.01$) were performed. The results were obtained from three independent experiments. PM/VT, pregnancy morbidity and vascular thrombosis.

by hypertension, endothelial dysfunction, and, in severe cases, thrombocytopenia.

In APS, the development of thrombosis is associated with reduced endothelial NO through the binding of aPL to the apolipoprotein E receptor 2 (ApoER2) by β2GPI, which leads to the protein phosphatase 2A (PP2A) activation that induces eNOS dephosphorylation (Sacharidou et al., 2018). Recently, the same proteins were detected in trophoblast with pathological functions, leading to pregnancy morbidity. The binding of aPL by ApoER2-stimulated PP2A, which reduced proliferation and trophoblastic migration associated with the development of preeclampsia for upregulation in hypoxia-inducible factor 1 and soluble endoglin (Chu et al., 2021). In APS, the development of pregnancy morbidity is related to endothelial activation and dysfunction that induce defective placental formation; however, the effect of aPL on trophoblast cells is vital (Abrahams et al., 2017; Quao et al., 2018). aPL reduce decidual endovascular trophoblast invasion (Sebire et al., 2002), leading to early recurrent miscarriages in APS (Abrahams et al., 2017; Jovanovic Krivokuca et al., 2017; Quao et al., 2018). Contrarily, aPL also induce pregnancy morbidity in the second and third trimester by inducing placental dysfunction that is evidenced by preeclampsia and/or intrauterine growth restriction (Antovic et al., 2018; Chu et al., 2021).

Hydroxychloroquine and Enoxaparin Restored Nitric Oxide Reduced by RI+ Immunoglobulin-G

Hydroxychloroquine (HCQ) and ENX modulate the endothelial dysfunction induced by RI+ IgG and β2GPI. ENX only modulated endothelial dysfunction generated by RI + IgG without β2GPI. Miranda et al. (2019) showed that HCQ restores eNOS phosphorylation decreased by aPL and thrombus formation *in vivo*. These authors found that HCQ reverses the increased tissue factor and decreased thrombomodulin induced by aPL in human aortic endothelial cells (Miranda et al., 2019). However, the previous study did not include different groups of patients, and the effects of HCQ on NO bioavailability through the blockade of NO-donors are unknown. Our study found the existence of the modulating effect of HCQ in patients with refractory primary APS with pregnancy morbidity and

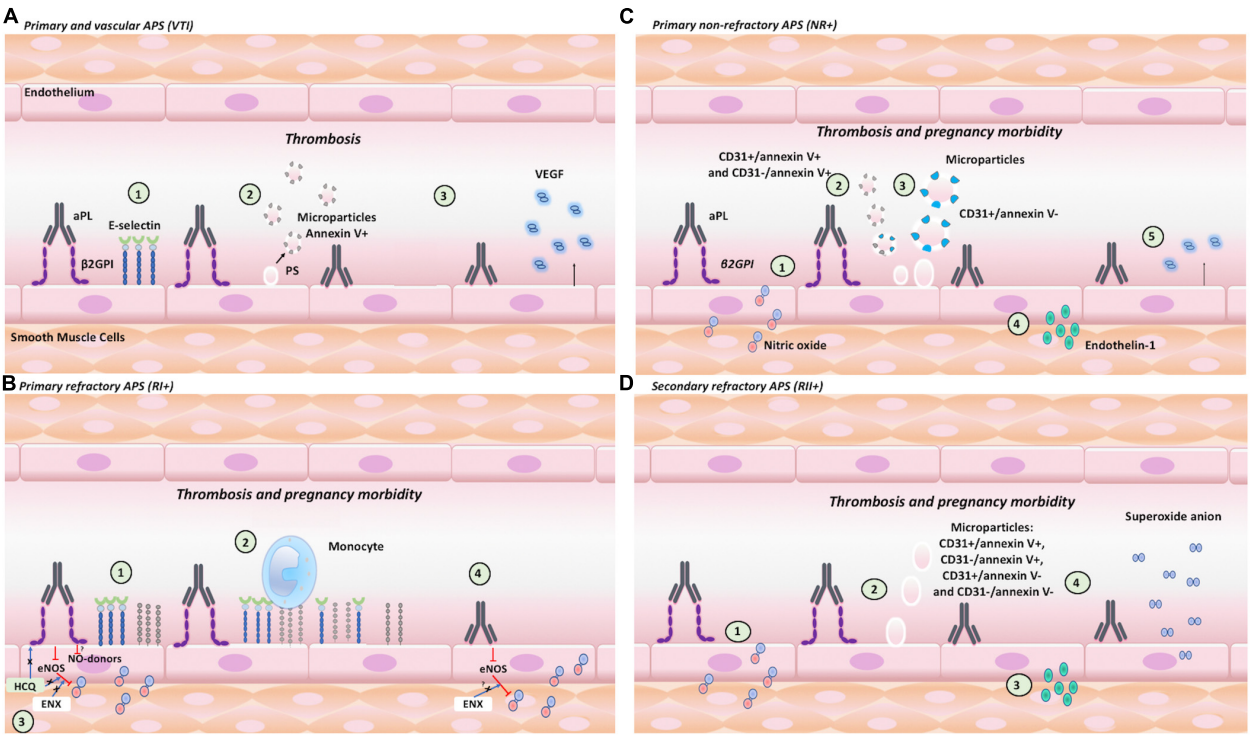

FIGURE 8 | Endothelial activation and dysfunction in APS: clinical manifestation association and role of β2GPI. The pathogenetic effects associated with endothelial activation induced by aPL with or without β2GPI contribute to thrombosis or pregnancy morbidity. **(A)** *VTI+ IgG:* (1) increased the expression of E-selectin; (2) generated positive annexin V procoagulant MPs of 0.5 μm; and (3) increased the VEGF generation. **(B)** *RI+ IgG:* (1) increased the expression of E-selectin and VCAM; (2) induced monocyte adhesion; (3) decreased NO bioavailability, which was modulated by HCQ and ENX; and (4) decreased NO bioavailability, which was modulated by ENX. **(C)** *NR+ IgG:* (1) decreased NO bioavailability; (2) increased the number of 0.5-μm procoagulant MPs (CD31+/annexinV+ and CD31-/annexinV+); (3) increased CD31+/annexinV, MP of 1 μm; (4) increased endothelin-1 production; and (5) reduced VEGF. **(D)** *RII+ IgG:* (1) decreased NO bioavailability; (2) increased MPs of 1 μm; (3) generated endothelin-1 production; and (4) induced oxidative stress (mitochondrial O_2^- production).

thrombosis (RI+). This modulating effect of HCQ is also explained by the disintegration that this drug induces on the phospholipid/β2GPI/aβ2GPI complexes (Rand et al., 2008). HCQ disintegrates the β2GPI/aPL complexes, preventing downstream pathological effects; it also modulates proinflammatory cytokine production, such as tumor necrosis factor-alpha, and mitigates the increase of HUVEC permeability induced by the serum from patients with preeclampsia (Rahman et al., 2020). Likewise, heparin binds to β2GPI in domain V through an interaction with Lys284, Lys286, and Lys287, and decreases the ability to recognize aPL aβ2GPI by reducing their prothrombotic activity (Guerin et al., 2002). Similar to HCQ, heparin increases the phosphorylation of eNOS, thus increasing NO (Li et al., 2020).

Increased Endothelin-1 and Vascular Endothelial Growth Factor Production Changes as an Indicator for Endothelial Dysfunction Associated With Antiphospholipid Syndrome Clinical Manifestations

Non-refractory primary obstetric APS IgG with β2GPI decreased NO bioavailability and VEGF production but increased endothelin-1 generation, which explains one of the triggering mechanisms of thrombosis and pregnancy morbidity and hypertension. Our results agree with previous findings in which pregnancy morbidity was associated with a decreased NO that induces endothelin-1 production (Saleh et al., 2016). Nevertheless, a VEGF reduction was related with enhancing this vasoconstrictor as a switch in the angiogenic mechanisms, associated with placental malformation, pregnancy morbidity, and other pathologies, such as cancer (Lankhorst et al., 2016). Despite this, IgG from patients with VTI and VTII increased the VEGF production as a possible thrombosis-inducing mechanism associated with intimal hyperplasia at the thrombus site (Williams et al., 2000).

Induction of Oxidative Stress Associated With Endothelial Dysfunction

On the other hand, RII+ IgG with β2GPI decreased NO bioavailability, but without this cofactor increased endothelin-1 and induced O_2^- production compared with the NR+ primary APS (NR+). Independent results of β2GPI indicate that this effect is generated by aCL without requiring this cofactor or other autoantibodies present in RII+ patients. Human monoclonal aCL injected into BALB/c mice was reported to

induce O_2^- production. However, these antibodies showed reactivity to β2GPI (Delgado Alves et al., 2005). Hence, the role of aCL in this event was unclear, but Simoncini et al. (2005) showed that after eliminating aβ2GPI, aPL from patients with APS-induced ROS production in endothelial cells compared with the NHS IgG control, suggesting the relevance of β2GPI-independent aCL. A few studies reported that the serum of patients with SLE, positive for anti-double-stranded DNA antibodies, induced intracellular ROS production and increased NADPH oxidase activity, which catalyzes O_2^- production (Didion and Faraci, 2002; Toral et al., 2017). However, patients were not classified by clinical manifestation in these previous studies. According to the results in this study, β2GPI-independent autoantibodies from patients with RII+ trigger thrombosis and pregnancy morbidity through endothelial oxidative stress, indicating a potential antioxidant therapy in patients with refractoriness to conventional treatment. These results indicate that not only β2GPI-independent aCL generate events associated with endothelial activation in these patients. RII+ IgG triggers endothelial activation and dysfunction, characterized by ROS production: O_2^- production reduces NO bioavailability, impairing vascular relaxation, which increases in endothelin-1 and enhances vasoconstriction (Silva et al., 2012).

Immunoglobulin-G From Patients With NR+ Obstetric Antiphospholipid Syndrome-Induced Endothelial Microparticles Production, Increasing Endothelial Activation and Dysfunction

Endothelial dysfunction is characterized by the outburst of microparticles (Amabile et al., 2005; Deng et al., 2017; Leite et al., 2020). IgG from patients with APS, presenting thrombosis with or without pregnancy morbidity, increased endothelial microparticles *in vitro* (Pericleous et al., 2013). Endothelial microparticles reportedly increased only in the plasma of patients with thrombotic APS compared with healthy controls and patients with obstetric APS (Breen et al., 2015). VTI IgG with β2GPI produced 0.5-μm microparticles positive for phosphatidylserine, considered as procoagulants. These microparticles induce coagulation by exposing phosphatidylserine, which activates tissue factor, a receptor for factor VIIa, and initiates the coagulation cascade through an extrinsic pathway (Owens and Mackman, 2011). Additionally, NR+ IgG increased the number of 0.5-μm procoagulant microparticles (CD31+/annexinV+ and CD31-/annexinV+) and a 1-μm CD31+ microparticle. RII+ IgG generates total microparticles of 1 μm. Microparticles of endothelial cells were found in patients with recurrent miscarriage, indicating damage and endothelial activation (Carp et al., 2004).

CONCLUSION

Our results suggest that endothelial activation and dysfunction in APS are seen in different contexts, and the induction mechanism varies according to the clinical characteristics of patients and the presence of aPL cofactors, such as β2GPI. HCQ only modulates endothelial dysfunction generated by RI+ IgG with β2GPI, and ENX modulates endothelial dysfunction generated by RI+ IgG with or without β2GPI. Additionally, we recommended that in future studies, patients with APS must be classified according to clinical manifestation and that these findings should be considered when using these drugs in patients with refractory APS, specifically primary (RI+).

STRENGTHS AND LIMITATIONS

In this study, the classification of patients by clinical manifestations and the addition of β2GPI to the HUVECs allowed us to understand the differences that aPL present in endothelial activation and dysfunction, representing an adequate methodology in the study of APS. However, the number of individuals in this study was limited, considering the difficulty in recruiting patients with autoimmunity well-characterized by the low prevalence of this type of disease even more with refractoriness. Our experimental design should be applied to a larger population of patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the University of Antioquia. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ÁC and MV generated the conception of this study. MV wrote the draft of the manuscript, performed the experiments, and analyzed the data. LP performed some experiments on expression adhesion molecules. MR, SS, RN-S, CE, JV, and ÁC contributed to the analysis and interpretation of data. All authors have reviewed and approved the manuscript.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.764702/full#supplementary-material

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Uvaol Prevents Group B *Streptococcus*-Induced Trophoblast Cells Inflammation and Possible Endothelial Dysfunction

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Group B *Streptococcus* (GBS) infection during pregnancy is involved in maternal sepsis, chorioamnionitis, prematurity, fetal infection, neonatal sepsis, and neurodevelopmental alterations. The GBS-induced chorioamnionitis leads to a plethora of immune and trophoblast cells alterations that could influence endothelial cells to respond differently to angiogenic mediators and alter placental vascular structure and function in pregnant women. In this context, preventive measures are needed to reduce such dysfunctions. As such, we evaluated the effects of a non-lethal exposure to inactivated GBS on trophoblast cells and chorionic villi explants, and if the treatment with uvaol would mitigate these effects. The concentration of 10^6 CFU of GBS was chosen since it was unable to reduce the HTR-8/SVneo cell line nor term chorionic villi explant viability. Raman spectroscopy of trophoblast cells showed significant alterations in their biochemical signature, mostly reverted by uvaol. GBS exposure increased HTR-8/SVneo cells IL-1β and IFN-γ production, phagocytosis, oxidative stress, and decreased trophoblast cell migration. The Ea.hy926 endothelial cell line produced angiopoietin-2, CXCL-8, EGF, FGF-b, IL-6, PlGF, sPECAM-1, and VEGF in culture. When co-cultured in invasion assay with HTR-8/SVneo trophoblast cells, the co-culture had increased production of angiopoietin-2, CXCL-8, FGF-b, and VEGF, while reduced sPECAM-1 and IL-6. GBS exposure led to increased CXCL-8 and IL-6 production, both prevented by uvaol. Chorionic villi explants followed the same patterns of production when exposed to GBS and response to uvaol treatment as well. These findings demonstrate that, even a non-lethal concentration of GBS causes placental

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inflammation and oxidative stress, reduces trophoblast invasion of endothelial cells, and increases CXCL-8 and IL-6, key factors that participate in vascular dysregulation observed in several diseases. Furthermore, uvaol treatment prevented most of the GBS-provoked changes. Hence, uvaol could prevent the harmful effects of GBS infection for both the mother and the fetus.

Keywords: Group B *Streptococcus*, infection, placenta, vascular dysfunction, trophoblast, uvaol

INTRODUCTION

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is a gram-positive bacterium responsible for a great number of maternal and fetal morbidity and mortality. This bacterium is part of the vaginal and/or gastrointestinal tract of 10–30% of pregnant women, where the colonization is usually asymptomatic. Nevertheless, GBS can cause chorioamnionitis, and it is involved in the development of maternal and neonatal sepsis, prematurity, and alterations in fetal neurodevelopment (van Dillen et al., 2010; Allard et al., 2018; Doster et al., 2018; Zhu et al., 2019). Group B *Streptococcus* infection is also involved in endothelial dysfunction (Beyrich et al., 2011; Doster et al., 2018), which is known to be linked to other harmful conditions for both the mother and the fetus, such as preeclampsia (Valencia-Ortega et al., 2019). Due to the severity of GBS infection, the European consensus was achieved to recommend intrapartum antimicrobial prophylaxis based on a universal GBS screening strategy using fast real-time testing (di Renzo et al., 2015). Recently, the American College of Obstetricians and Gynecologists (ACOG) has also published updated guidelines to recommend GBS screening and prophylaxis, which replaced the previous guidelines from 2010 (Prevention of Group B Streptococcal Early-Onset Disease in Newborns | ACOG, 2010). Nonetheless, Latin American countries still do not have effective guidelines, and a recent study has shown that less than 15% of pregnant women are, indeed, screened for GBS, except Uruguay, where 65% of them are screened. In the latter country, GBS colonization was found in 18.5% of women, while the highest prevalence was found in black women, older women, and women without primary education (HogenEsch et al., 2021). Considering these data, it is important to highlight the deficiency of data for most Latin American countries. As an example, the biggest and more populated country of the region, Brazil, does not even have GBS screening and prophylaxis guidelines consensus in its National Healthcare Public System (Nascimento et al., 2019).

Although prophylaxis with antibiotics is proven effective (Vornhagen et al., 2017), a preventive alternative is inexistent at the moment. In this context, one of the biggest advances in obstetrics was the introduction of nutraceuticals to prevent fetal malformations or maternal conditions. A healthy diet is linked to reduced numbers of non-desirable pregnancy outcomes, such as abortion, prematurity, preeclampsia, gestational *diabetes mellitus*, intrauterine growth restriction (IUGR), and others (Wattar et al., 2019). Amongst all diets, it is well-known the benefits from the Mediterranean diet on poor pregnancy outcomes, particularly the ingestion of olives (*Europea olea*) or olive oil, which are potent anti-inflammatory foods (de La Torre et al., 2019; Wattar et al., 2019). One of its compounds, the pentacyclic triterpene uvaol (urs-12-ene-3,28-diol), is described to prevent trophoblast cell death, cytoskeleton changes-reduced membrane elasticity, and the increase of IL-1β, IL-2, and IFN-γ from GBS at 10^8 CFU incubation (Botelho et al., 2019). Moreover, uvaol was also described to improve cell migration and tube formation in endothelial cells through PKA and p38-MAPK signaling pathways, increasing wound healing and angiogenesis (Carmo et al., 2020).

Hence, we believe that GBS-induced chorioamnionitis could lead to a plethora of immune and trophoblast cells alterations, which, in turn, influence endothelial cell function in pregnant women. As such, we evaluated the effects of a non-lethal exposure of inactivated GBS on trophoblast cells and chorionic villi explants, the repercussions on endothelial cells, and if the treatment with uvaol would mitigate these effects.

MATERIALS AND METHODS

Cell Culture

The first trimester-derived extravillous trophoblast cell line HTR-8/Svneo, and the endothelial cell line EaHy-926 was separately cultured in the DMEM/F12 medium (Merck/Sigma-Aldrich, St. Louis, MO, United States) with 10% fetal bovine serum (FBS) and 2-mM L-glutamine (all from Thermo Fisher Scientific, Waltham, MA, United States) at 37°C and 5% CO_2. HTR-8/SVneo cells were subcultured when ∼70% confluence was achieved, whereas EaHy-926 with ∼90% confluence. Cells were reseeded at 10^4 cells/cm^2.

Collection and Culture of Placental Chorionic Villi Explants

The samples were collected at the Obstetrics Service of HUPAA/UFAL from healthy term pregnancies (37–40 weeks) with cesarean deliveries, under an approved ethics committee protocol. A total of eight placentas were obtained from 18- to 35-year-old women, with no current gestational, autoimmune, and infectious diseases, and genetic disorders. The study was approved by the Ethics Committee (52237915.5.0000.5013), and ethical considerations were based on the use of the material for scientific purposes, with the confidentiality of the patient identity and without constraint from the institutions or people involved. After the placental collection, two cotyledons were washed with saline solution and carefully scraped with a scalpel blade to obtain the terminal villi in a glass Petri dish. Samples were grown in the DMEM/F12 medium, and, after 24 h, the media was renewed.

Uvaol Treatment

Uvaol (Merck/Sigma-Aldrich) was diluted in 1% dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS) (v/v). Treatment with uvaol or only its vehicle was performed 24 h from the cell plating. The chosen concentration for HTR-8/SVneo cells was 10 μM, which we previously showed as a concentration that does not affect cell viability (Botelho et al., 2019), and, for placental explants, uvaol was used at 50 μM based on the following 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) results.

Group B *Streptococcus* Incubation

The β-hemolytic GBS serotype Ia strain (#16955) that we used has had its isolation, growth, and inactivation methods described previously (Bergeron et al., 2013). Briefly, GBS was isolated at CHUS Fleurimont from a pregnant patient and grown in brain-heart infusion (BHI) broth for 15 h at 37°C. The culture was diluted at 1:100 and further grown until the mid-exponential phase. Titration was performed by counting colony-forming units (CFU) on BHI agar plates. The bacteria were harvested by centrifugation at 3,220 g and inactivated in a solution of 10% formaldehyde for 24 h. Afterward, GBS was washed thoroughly with saline solution. Inactivated GBS was harvested and suspended in sterile suspension at 10^9 CFU/100 μL; 50 μL of the inactivated suspension was plated in duplicate on BHI agar and incubated at 37°C overnight to verify that all bacteria were killed. For the *in vitro* incubation experiments, GBS at 10^6 CFU/ml was used. In experiments of cell death, *in vitro* wound healing assay, and 3D co-culture endothelial invasion test, GBS was incubated for 48 h. Some phagocytosis assays had GBS incubation for 2 h. Other experiments had 24 h incubation time.

The MTT Cell Viability Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to verify the possible cytotoxicity of uvaol and GBS. As such, HTR-8/SVneo cells or placental explants were treated with uvaol for 1 h before GBS incubation or untreated, followed by GBS incubation, and further cultured in DMEM-F12 media supplemented with 10% FBS for 24 h. The medium was replaced with a fresh culture medium, containing 5 mg/ml of MTT, and the supernatant was discarded after a 4-h incubation period at 37°C, followed by the addition of DMSO solution. The absorbance of the solubilized MTT formazan product was spectrophotometrically measured at 540 nm. The percentage viability was determined about control [(absorbance of treated cells/absorbance of untreated cells) × 100].

Measure of Cell Death

Cell death was evaluated using the Annexin V-fluorescein (FITC)/Propidium Iodide (PI) detection kit (BD Biosciences) according to the descriptions of the manufacturer. In summary, 3×10^5 cells were plated, and the cells were analyzed after 24 h and 48 h after uvaol treatment and GBS incubation. After cell detachment with 25% trypsin solution (v/v), cells were incubated with BD Binding Buffer (BD Biosciences, Franklin Lakes, NJ, United States) and then incubated with 5 μL of annexin V and 5 μL of PI in the dark for 20 min. The results were acquired with the flow cytometer BD FACS Canto II (BD Biosciences) and analyzed using the software FlowJo v.10.7.2 (BD Biosciences).

Raman Spectral Measurements

Raman spectra were measured using an XploRA spectrometer (Horiba, Japan), coupled to an optical microscope (BXFM, Olympus, Japan) and equipped with a 532-nm laser that was focused on the nucleus of the cells through a 100 × objective (NA = 0.9). The same objective lens was used for collecting Raman-scattered light after interaction with the sample, in backscattering geometry. The frequency calibration was set by reference to the 520-cm^{-1} vibrational bands of a silicon wafer. Under the same conditions, 60 cell spectra captured in three different experiments for each group were measured in the spectral range of 600–1,800 cm^{-1}. To minimize laser-induced heating of the specimens, low-power irradiation at the sample surface was used, around 5 mW, during a short exposure time (3-s laser exposure for five accumulations). The diffraction grating used had 1,200 lines/mm, which yielded a spectral resolution of 1.5 cm cm^{-1}.

Data Preprocessing and Spectral Analysis

Before conducting the spectral analysis, all spectra were smoothed, background-adjusted, and normalized using an algorithm implemented in MatLab software (Mathworks, Naticks, MA, United States). As such, the external noises were suppressed, and the useful information about the biochemical composition was enhanced. After removing the background fluorescence from the spectra, Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA) were performed to evaluate the spectral variability in the dataset. Each cell group was subjected to 3D-PCA and an unsupervised HCA with Euclidean distances. All multivariate statistical analyses were implemented in MatLab software. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to analyze the spectral differences in the samples and to predict which class each sample belongs to. This multivariate statistical analysis was performed through the SIMCA 17 software (Umetrics, Umea, Sweden). Moreover, Raman individual peaks with statistically significant fluctuations were isolated to compare. For further analysis, the extracted band intensities were imported to GraphPad Prism software (Graph Pad Software Inc., San Diego, CA, United States) to apply one-way ANOVA and statistical analysis.

Phagocytosis Assay

HTR-8/SVneo cells were cultured at 5×10^4 in 24-well plates containing glass coverslips. After 24 h, they were treated with 10-μM uvaol for 1 h and stimulated with inactivated or live GBS at 10^6 CFU for further 2 h. Afterward, cultures were washed with PBS, fixed with ice-cold methanol, and stained with Giemsa staining. The coverslips were mounted with Entellan (Sigma-Aldritch) on histological slides and examined under a Nikon Eclipse 50i optical microscope (Nikon, Japan) in

a 100 × objective with immersion oil. The percentage of phagocytosis was determined by the presence of GBS inside trophoblast cells cytoplasm [(number of cells with GBS in the cytoplasm/total number of cells) × 100]. Additionally, groups to analyze the influence of recombinant addition of 100 UI/ml IFN-γ (Merck/Sigma-Aldrich; Albieri et al., 2005) on phagocytosis were performed where cells were treated for 24 h with IFN-γ before GBS incubation or 24 h after GBS incubation.

Mitochondrial Reactive Oxygen Species Production Assay

After the previously described treatments, MitoSOX red (Thermo Fisher Scientific, Waltham, MA, United States) staining was incubated in living cells, following the conditions of the manufacturer. After 30 min, cells were fixed with 4% paraformaldehyde in PBS (v/v), washed with PBS, stained with 4',6-diamidino-2-phenylindole (DAP-I), and mounted with PBS/glycerol (1:9, v/v) under glass slides. The results were visualized with a fluorescence microscope Nikon DS-Ri1 (Nikon). Images were acquired using the DP2-BSW software (Nikon). The percentage of cells with Mitochondrial Reactive Oxygen Species (mtROS) was determined by [(number of cells positively stained by MitoSOX/total number of cells) × 100].

Cytoskeleton Evaluation of F-Actin Polymerization

HTR-8/SVneo cells were plated at 3×10^5 cells, and, after 24 h, uvaol was added 1 h before GBS incubation and maintained for 24 h. The cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with.1% Triton X-100 in PBS (v/v). Phalloidin-fluorescein (FITC)-conjugated staining (1:100; Abcam, Cambridge, United Kingdom) was added for 1 h, and nuclei were stained with DAP-I. Cells were mounted with PBS/glycerol (1:9, v/v) under glass slides, and the results were visualized with fluorescence microscope Nikon DS-Ri1 (Nikon). Images were acquired using the DP2-BSW software (Nikon).

In vitro Wound-Healing Assay

HTR-8/SVneo cells were plated at 3×10^5 cells until reaching ∼ 100% confluence. Afterward, scratches of equal size were performed with a 200-μL pipette tip. Cells were treated with 10-μg/ml mitomycin C (Sigma-Aldritch) for 2 h. Uvaol was added 1 h before GBS incubation and maintained for 48 h. Pictures were taken after 12, 24, 36, and 48 h (400 × magnification). The distance from the scratch borders was measured using the ImageJ free software (NIH, Bethesda, MD, EUA), and the closure percentage of each point was calculated.

Three-Dimensional Vascular Invasion Assay

The Three-Dimensional (3D) vascular invasion assay was performed in transwell chambers using 24-well fitted inserts with 8-μm pore size. A mixture of 8×10^5 EaHy-926 endothelial cells/well in 15 μL of Matrigel (Sigma; 1:1 v/v in DMEM/F12) was plated on the upper chamber, and DMEM/F12 was added in both compartments after polymerization. The cells were kept until ∼100% confluence was achieved. Afterward, HTR-8/SVneo cells were stained with 1 μM of carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific) and kept in the dark for 10 min. The CFSE-stained HTR-8/SVneo cells were placed at 2×10^5 cells/well on top of the previous mixture of Matrigel and EaHy-926 cells, and supplemented DMEM/F12 was added in both compartments (5% FBS in an upper compartment with 10-μM uvaol, and 20% FBS in the lower compartment). After 1 h, GBS was added to the upper compartment. After 48 h, non-invading cells from the upper compartment were removed with a cotton swab, supernatants were collected, and the membranes were fixed with ice-cold methanol. Nuclei were stained with DAP-I, and the cut membranes were mounted with PBS/glycerol (1:9, v/v) under glass slides. The results were visualized with fluorescence microscope Nikon DS-Ri1 (Nikon, Japan), and images were acquired at 400 × magnification using the DP2-BSW software (Nikon).

Supernatant Detection of Cytokines and Angiogenic Factors

All collected supernatants were analyzed for IL-1β using an ELISA kit (Sigma-Aldrich) following the instructions of the manufacturer. Tetramethylbenzidine and hydrogen peroxide were used as substrates in the peroxidase reaction, and plates were read at 450 nm using a Magpix system (Sigma-Aldrich). The supernatants from HTR-8/SVneo cells were evaluated using the LEGENDplex human Th1/Th2 Panel (BioLegend, San Diego, CA, United States) kit for detecting IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNF-α, and IFN-γ levels. The supernatants from HTR-8/SVneo cells, the 3D vascular invasion assay, and from placental chorionic villi were analyzed with the LEGENDplex human Angiogenic Panel (BioLegend) kit for detecting IL-6, CXCL-8, Angiopoietin-1, Angiopoietin-2, fibroblast growth factor basic (FGF-b), epidermal growth factor (EGF), soluble PECAM-1 (sPECAM-1), placental growth factor (PlGF), vascular endothelial growth factor (VEGF), and TNF-α. Both LEGENDplex kits were employed according to the instructions of the manufacturer. The cytokines and angiogenic factors were detected using FACS Canto II (BD Biosciences), and the mean fluorescence intensity (MFI) from the samples was converted to pg/ml using the LEGENDplex v.8.0 software (BioLegend).

Statistical Analysis

To show that all data were normally distributed, a Kolmogorov-Smirnov test was performed. Matlab software was used for spectral analysis, and PCA and HCA were used for spectral multivariate analysis. Classification of samples by OPLS-DA was directly reflected by the principal component scores of the OPLS-DA model, and the robustness of this model was assessed based on the following parameters: R2X (cum), the cumulative sum of squares of all x-variables explained by all extracted components; R2Y(cum), the cumulative sum of squares of all y-variables explained by all extracted components; and Q2(cum), the fraction of all x-variables and y-variables that can be predicted for the extracted component. Other results were analyzed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA,

United States) with one-way ANOVA using Dunnett or Tukey *post hoc* test, and cell death was analyzed with two-way ANOVA using Bonferroni *post hoc* test. The minimal level of significance for all experiments was set at $p < 0.05$. The results are depicted as the mean ± standard error of the mean (SEM).

RESULTS

Inactivated Group B *Streptococcus* Does Not Change Placental Explants and Trophoblast Cell Viability

Placental explants incubated with GBS at 10^6 CFU alone or after uvaol treatment had no statistically significant changes in overall viability concerning control (**Figure 1A**). A similar result was found in HTR-8/SVneo cells, with no changes in their viability (**Figure 1B**). To assess if GBS at 10^6 CFU, indeed, did not induce cell death, we analyzed the Annexin V and Propidium Iodide (PI) staining of trophoblast cells by flow cytometry. As result, no differences were observed, comparing all analyzed groups, nor when different time points (24 and 48 h) were evaluated (**Figure 1C**).

Raman Spectra Classification and Analysis

Through the Raman spectra, it was possible to identify the most important spectral differences among trophoblast cell groups. To perform spectral data analysis and classification, the Raman spectra were divided into three groups: (i) cells without treatment (control); (ii) cells treated with uvaol (uvaol); (iii) cells incubated with GBS at 10^6 CFU (GBS); and (iv) cells treated with uvaol and incubated with GBS (uvaol + GBS). A total of 60 cell spectra were measured for each group, from three independent experiments, and **Figure 2A** shows the average Raman spectra of the analyzed groups. We focused on all measurements over cell nuclei, even though Raman spectroscopy can be performed at any cell portion. Nonetheless, we found that HTR-8SV/neo cells were thin at the cell periphery, and the glass surface below greatly interfered with the readings. Moreover, the standardization of the region reduces variations that might be observed if random cell places were measured otherwise (Moor et al., 2018; Arend et al., 2020; Tiwari et al., 2020). A three-dimensional plot was constructed with combinations of sets of scores of the first three PCs as well as the corresponding plots of the PC1 and PC2 loadings, used for the determination of the differentiation capability of PCA and identification of significant Raman features (**Figures 2B,C**). The PC loadings are representative of the biochemical differences between cell groups and are responsible for differentiating the spectra in the score plot of PC. Indeed, the PC loadings effectively carry all the important information of the spectra and have a spectral dimension, where positive and negative peaks can be observed. In the PCA, loadings of PC1 indicated positive correlations of Raman bands at 1,452 and 1,667 cm^{-1}, and negative correlations at 725, 915, 1,028, and 1,226 cm^{-1}. The PC2 loadings indicated positive correlations in 1,091, 1,301, and 1,437 cm^{-1}, whereas negative correlations at 1,041 cm^{-1}. The PC3 loadings indicated positive correlations at 707, 1,442, and 1,554 cm^{-1}, whereas negative correlations at 788, 1,096, and 1,488 cm^{-1} (**Figure 2B**). As such, the first

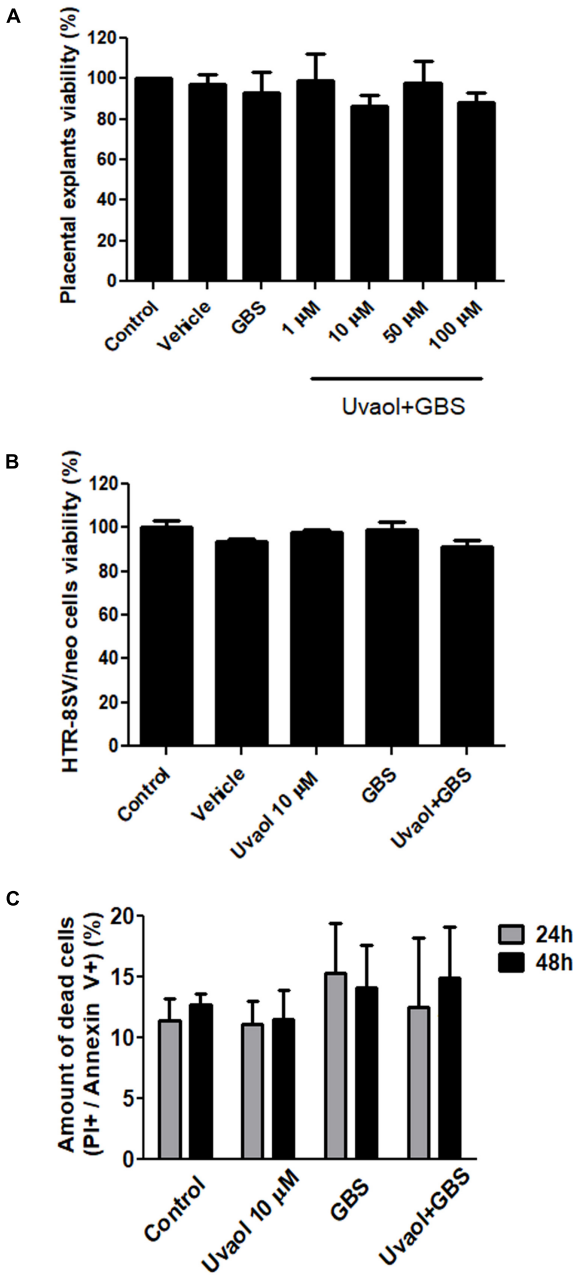

FIGURE 1 | Uvaol does not affect cells and explant viability. **(A)** The term chorionic villi explants were treated with DMSO (vehicle), GBS at 10^6 CFU (GBS), or with uvaol at 1, 10, 50, and 100 μM for 24 h and analyzed by MTT assay. **(B)** The HTR-8/SVneo cells were treated with vehicle, 10-μM uvaol, GBS at 10^6 CFU or both for 24 h, and analyzed by MTT assay. **(C)** Cells were treated for 24 h and 48 h, and annexin V/PI staining was analyzed through flow cytometry. Bar graphs represent mean values ± S.E.M.; $n = 4$ in triplicate in panel **(A,B)**, and $n = 3$ in duplicate in panel **(C)**. One-way ANOVA with Tukey *post hoc* test was used in panel **(A,B)**, whereas two-way ANOVA with Bonferroni *post hoc* test was used in panel **(C)**.

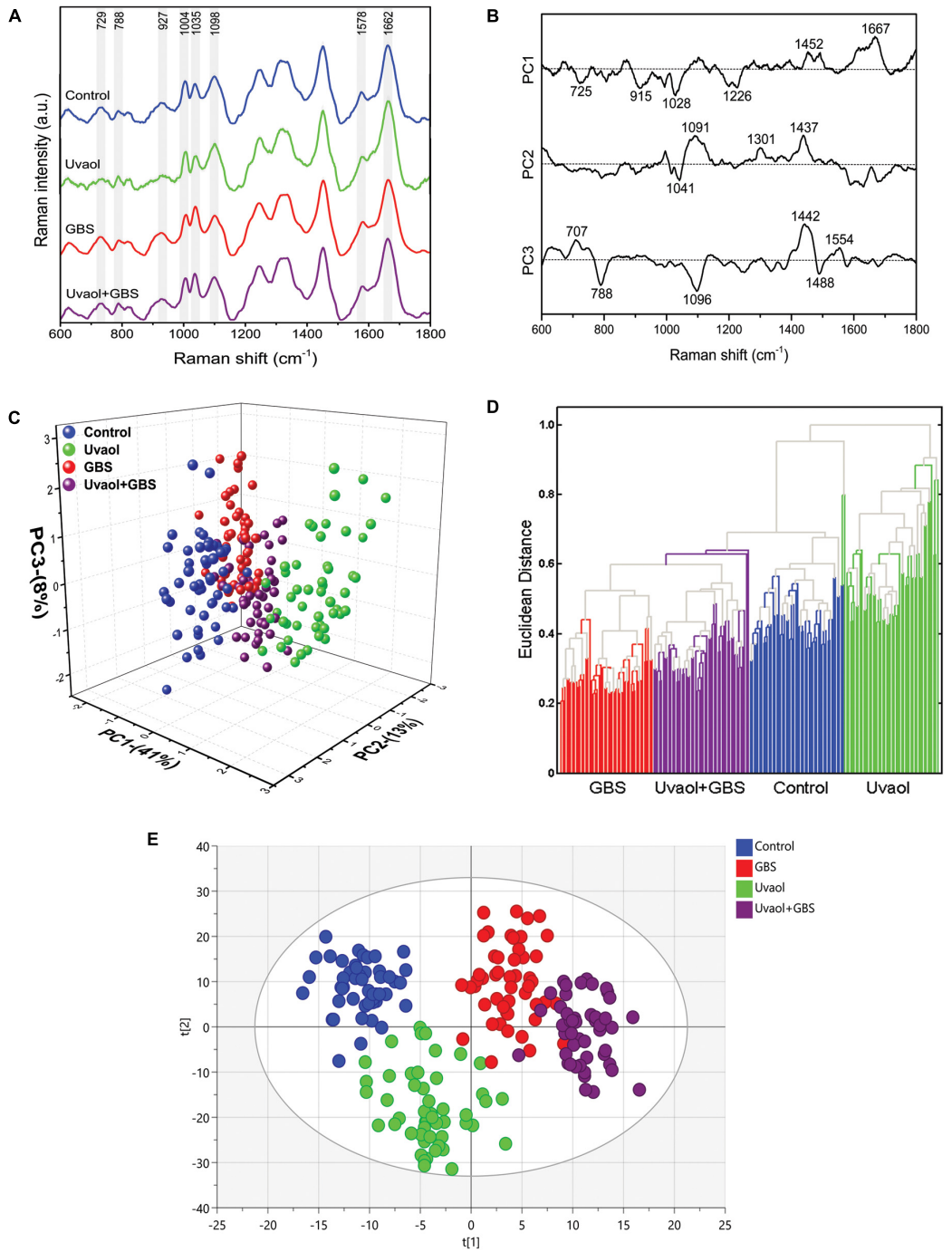

FIGURE 2 | GBS and uvaol change trophoblast cells biochemical signature. **(A)** The Raman spectra represent the averages of 50 cells for each group (Control, uvaol 10 μM, GBS at 10^6 CFU, and uvaol with GBS) in the fingerprint region (600–1,800 cm^{-1}). The shaded areas correspond to the Raman bands where the main variances observed in the average cell spectra occurred; **(B)** Loadings of PC1, PC2, and PC3 for Control, uvaol, GBS, and uvaol + GBS groups; **(C)** A three-dimensional PCA score plot; **(D)** HCA dendrogram for all analyzed groups; **(E)** A two-dimensional OPLS-DA score plot of the Raman spectra for the Control, uvaol, GBS, and uvaol + GBS groups. The four groups were relatively well-discriminated along the t[1] axis, as well along the t[2] axis. *N* = 3.

three PCs explained 62% of the variance of the original data set, with PC1 describing 41%, PC2 describing 13%, and PC3 describing 8% of the total variance, with principal component analysis (PCA) clearly dividing cells into four different clusters matching their groups (**Figure 2C**). Furthermore, hierarchical cluster analysis (HCA) was used for cell discrimination, and four separated clusters could be easily observed, each one representing a group, distinctively aggregated, with the GBS group being

the most distant from the control group (**Figure 2D**). The clusters showed a clear separation with no mixture of cells from different groups, corroborating the PCA and suggesting that uvaol, indeed, prevents certain biochemical changes induced by GBS (**Figure 2D**). We also employed the OPLS-DA, as it can separate predictive from non-predictive (orthogonal) variation to highlight the differences among the analyzed groups. The samples were separated with minimal overlap in the score scatter plot along the X and Y axes (**Figure 2E**) at a 95% confidence interval. The OPLS-DA of the Raman spectra resulted in the following specific parameters: R2X(cum) = 0.521, R2Y(cum) = 0.860, and Q2(cum) = 0.812 ($p < 0.05$). As such, the obtained parameters indicated that the OPLS-DA not only had discrimination power—they also indicated predictive precision of 86% to assign an unknown sample to one of the four analyzed groups.

Raman Spectrum Assignment of Trophoblast Cells

Based on the most significant changes, Raman band assignments and contributions used in the interpretation of spectral features were based on the published literature (Gelder et al., 2007; Movasaghi et al., 2007; Başar et al., 2012; Talari et al., 2014; Czamara et al., 2015; Surmacki et al., 2015; González-Solís et al., 2018; Keleştemur et al., 2018; Balan et al., 2019; Casal-Beiroa et al., 2021) and detailed in **Table 1**. Analyzing only uvaol treatment on trophoblast cells, we could observe that the Raman bands in 725, 729, 915, 927, 1,004, 1,035, 1,041, 1,091, 1,226, 1,301, and 1,437 cm^{-1} had reduced intensities in comparison to the control group, whereas the 788, 1,028, 1,452, 1,488, 1,554, 1,578, 1,662, and 1,667 cm^{-1} had increased intensities. Regarding GBS group differences in comparison to control, the bands in 1,004, 1,091, 1,096, 1,098, 1,301, 1,437, 1,442, 1,488, 1,662, and 1,667 cm^{-1} were reduced, while the bands in 725, 729, 788, 915, 1,028, 1,035, 1,041, 1,226, and 1,578 were increased. Interestingly, several of the bands reduced or increased in the uvaol group had the opposite result in the GBS group, which indicates that uvaol produces several biochemical alterations that could prevent trophoblast cells from the GBS opposite effects, or maybe help these cells to counter the negative effects risen from GBS contact. Consonantly, when we compared the Uvaol + GBS group with the GBS group, from the 20 changed Raman bands, uvaol prevented the alterations from 11 bands (725, 729, 915,

TABLE 1 | Raman bands position and their respective assignments and contributions.

Raman band position [cm^{-1}]	Band assignment	Contributions	Uvaol differences to control (*P*-values)	GBS differences to control (*P*-values)	Uvaol + GBS differences to GBS (*P*-values)
707	ν(C-S)	Methionine	NS	NS	NS
725	Ring breathing modes	Adenine	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.01$)	Reduced intensity ($p < 0.01$)
729	Ring breathing modes	Adenine	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.01$)	Reduced intensity ($p < 0.01$)
788	υ(O-P-O)	DNA	Increased intensity ($p < 0.01$)	Increased intensity ($p < 0.01$)	Increased intensity ($p < 0.05$)
915	βCH	RNA (Ribose)	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.01$)
927	υ(C–C)	CoA, Proline, Valine	Reduced intensity ($p < 0.001$)	NS	NS
1004	Ring breathing modes	Phenylalanine	Reduced intensity ($p < 0.001$)	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)
1028	βCH	Phenylalanine	Increased intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.001$)
1035	υ(C-C)	Amide I	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)	NS
1041	υ(C-O-C)	Glycosaminoglycans, Glutathione	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.001$)
1091	υ(C–C)	Myristic acid	Reduced intensity ($p < 0.01$)	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)
1096	υ_s(O-P-O), (PO_2^-)	Membrane phospholipids, DNA	NS	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.001$)
1098	$\upsilon_s(PO_2^-)$	DNA	NS	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.01$)
1226	υ(C-N)	Amide III (β-sheet)	Reduced intensity ($p < 0.001$)	Increased intensity ($p < 0.05$)	NS
1301	τCH_2	Fatty acids	Reduced intensity ($p < 0.01$)	Reduced intensity ($p < 0.001$)	NS
1437	αCH_3,αCH_2	Fatty acids, Sphingomyelin	Reduced intensity ($p < 0.01$)	Reduced intensity ($p < 0.001$)	NS
1442	αCH_3,αCH_2	Lipids	NS	Reduced intensity ($p < 0.001$)	NS
1452	δCH_2, δCH_3	Proteins	Increased intensity ($p < 0.05$)	NS	NS
1488	NH_3^+	Collagens	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.001$)	NS
1554	υ(C = C)	Amide II	NS	NS	NS
1578	N_3	Purines	Increased intensity ($p < 0.001$)	Increased intensity ($p < 0.01$)	NS
1662	υ_s(C = C)	Cholesterols, Amide I (β-sheet)	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.01$)	Increased intensity ($p < 0.001$)
1667	υ(C = C)	Amide I (α-helix), Proteins	Increased intensity ($p < 0.001$)	Reduced intensity ($p < 0.05$)	Increased intensity ($p < 0.001$)

NS non-significant; α- scissoring, β- bending, δ- deformation, τ- twisting, υ- stretching, υs- symetric stretching. Based on: Gelder et al., 2007; Movasaghi et al., 2007; Başar et al., 2012; Talari et al., 2014; Czamara et al., 2015; Surmacki et al., 2015; González-Solís et al., 2018; Keleştemur et al., 2018; Balan et al., 2019; Casal-Beiroa et al., 2021.

1,004, 1,028, 1,041, 1,091, 1,096, 1,098, 1,662, and 1,667 cm^{-1}), with a synergic effect of only one band (788 cm^{-1}). Regarding the 11 bands that uvaol helped to maintain after GBS incubation, they were mainly related to DNA, RNA, phenylalanine, glutathione, glycosaminoglycans, myristic acid, membrane phospholipids, cholesterols, and amide I. Nevertheless, the bands in 1,035, 1,226, 1,301, 1,437, 1,442, 1,452, 1,488, and 1,578 cm^{-1} were mainly related to amide I, amide III, fatty acids, lipids, proteins, and purines, which were altered by the GBS, although uvaol treatment was not able to prevent these changes. Altogether, the spectral analysis of the described bands can lead to the observation that a non-lethal concentration of GBS strongly modifies the biochemical composition of trophoblast cells, while uvaol prevented half of these changes.

Uvaol Prevents IL-1β and IFN-γ Secretion Triggered by Group B *Streptococcus*

Cytokine production by trophoblast cells was analyzed after 10^6 CFU of inactive GBS or with prior uvaol treatment. IL-10 had undetectable levels in all groups. IL-1β secretion increased in the GBS group compared to Control (10.8 ± 5.3 pg/ml to 27.6 ± 4.03 pg/ml; $p < 0.05$), whereas uvaol prevented this effect (12.87 ± 2.85 pg/ml; $p < 0.05$) (**Figure 3A**). IL-2 secretion was unchanged by GBS, but uvaol alone increased its production compared to Control (247.2 ± 88.08 pg/ml to 636 ± 82.98 pg/ml; $p < 0.01$) (**Figure 3B**). IL-4 secretion was unchanged in all analyzed conditions (**Figure 3C**), and uvaol increased IL-5 secretion compared to Control (120.01 ± 4.56 pg/ml to 178.8 ± 27.44 pg/ml; $p < 0.05$) (**Figure 3D**). Regarding IL-6, CXCL-8, and IL-13, they were not affected by any treatment (**Figures 3E–G**). Regarding IFN-γ secretion, it was remarkably increased by GBS exposure (206.2 ± 15.72 pg/ml to 2,870 ± 371.7 pg/ml; $p < 0.001$), while, in the Uvaol + GBS group, IFN-γ secretion was partially halted in relation to the GBS group (1,640.1 ± 654.5 pg/ml; $p < 0.05$) (**Figure 3H**). Lastly, TNF-α levels were unchanged in all groups (**Figure 3I**). Therefore, GBS increased IL-1β and IFN-γ in trophoblast cells, indicating a characteristic inflammatory state in our trophoblast cell culture that was mostly prevented by the addition of uvaol.

Uvaol Reduces Trophoblast Cell Phagocytosis and Oxidative Stress

Trophoblast cell phagocytosis of GBS and oxidative stress generation was also analyzed. As a result, trophoblast cells, indeed, phagocytized the inactive GBS (**Figures 4A–C**). With the addition of uvaol, the number of cells that had phagocyted inactivated GBS after 2 h was greatly reduced from 52.50 ± 1.04 to 31 ± 1.08% ($p = 0.0004$) (**Figures 4A–D**). With the addition of IFN-γ before GBS 2-h incubation, no changes were found (**Figure 4E**). Analyzing the 24-h incubation of GBS, uvaol could still reduce the phagocytosis amount (63.37 ± 4% to 37.92 ± 6.06%; $p < 0.05$) (**Figure 4F**), and IFN-γ addition prior or after GBS incubation could not increase phagocytosis (**Figure 4G**). Moreover, using live GBS, trophoblast cells phagocyted much less GBS (20.55 ± 0.27%), although uvaol had a similar effect on further reduction (9.29 ± 0.57%; $p = 0.0037$) (**Supplementary Figure 1**). Regarding mitochondrial-derived reactive oxygen species (mtROS), it was scarcely detected in the control and uvaol groups (**Figures 4H,I,N**). Group B *Streptococcus* incubation dramatically increased mtROS compared to control (14.24 ± 4.41% to 85.55 ± 7.56%; $p < 0.001$), whereas uvaol was able to, at least, partially prevent mtROS production (36.62 ± 10.02%; $p < 0.01$) (**Figures 4H–N**).

Uvaol Prevents Reduced Trophoblast Cell Motility Induced by Group B *Streptococcus*

Phalloidin staining was performed to evaluate GBS effects on trophoblast cells. Although Control and uvaol groups were characterized by a majority of fusiform cells with filopodia and F-actin stress fibers, cells in the GBS group cells were roundish and with fewer filopodia, while uvaol treatment partially prevented these changes (**Figure 5A**). Trophoblast cells also had their migration evaluated with *in vitro* scratch assay for 48 h. The scratch closure rates of the Control group were 23.92% ± 2.47% after 12 h, 46.54% ± 3.52% after 24 h, 53.4% ± 5.75% after 36 h, and 61.89% ± 6.64% after 48 h (**Figure 5B**). Uvaol addition has remained unchanged the wound closure rate. Nevertheless, the GBS group had a significant reduction in the scratch closure at 12 h, 24 h, and 48 h compared to Control (respectively, $p < 0.01$, $p < 0.01$, and $p < 0.05$), since GBS closure rates were 7.44% ± 1.97% after 12 h, 24.2% ± 3.84% after 24 h, 34.55% ± 5.07% after 36 h, and 23.29% ± 7.38% after 48 h (**Figure 5B**). Uvaol addition before GBS incubation prevented the migratory reduction induced by GBS: 24.37% ± 2.99% after 12 h ($p < 0.01$), 39.44% ± 2.6% after 24 h ($p < 0.05$), 44.73% ± 3.77% after 36 h, and 46.41% ± 5.37% after 48 h ($p < 0.05$) compared to the GBS group (**Figure 5B**).

Uvaol Prevents Group B *Streptococcus*-Induced Reduction of Trophoblast Invasion in a Three-Dimensional Model of Coculture With Endothelial Cells

Since trophoblast cells must invade and remodel uterine spiral arteries during pregnancy, the interface of the trophoblast-endothelial cells was analyzed with a 3D coculture vascular invasion assay. As such, control invasion was set to 100%. Uvaol unchanged invasion rates. Nevertheless, the GBS group reduced by half the number of invading cells, where only 47.54% ± 8.28% cells successfully invaded through the endothelial cells and Matrigel mixture ($p < 0.001$) (**Figures 5C,D**). When uvaol was added before GBS, the group had 77.22% ± 5.28% of invaded cells ($p < 0.05$), representing important prevention of GBS-induced changes (**Figures 5C,D**).

Invading Trophoblast Cells Change Endothelial Cell Production of Vasoactive Molecules

Endothelial cells produced almost all vasoactive factors measured here, except Angiopoietin-1 (**Figure 6A**). Angiopoietin-2

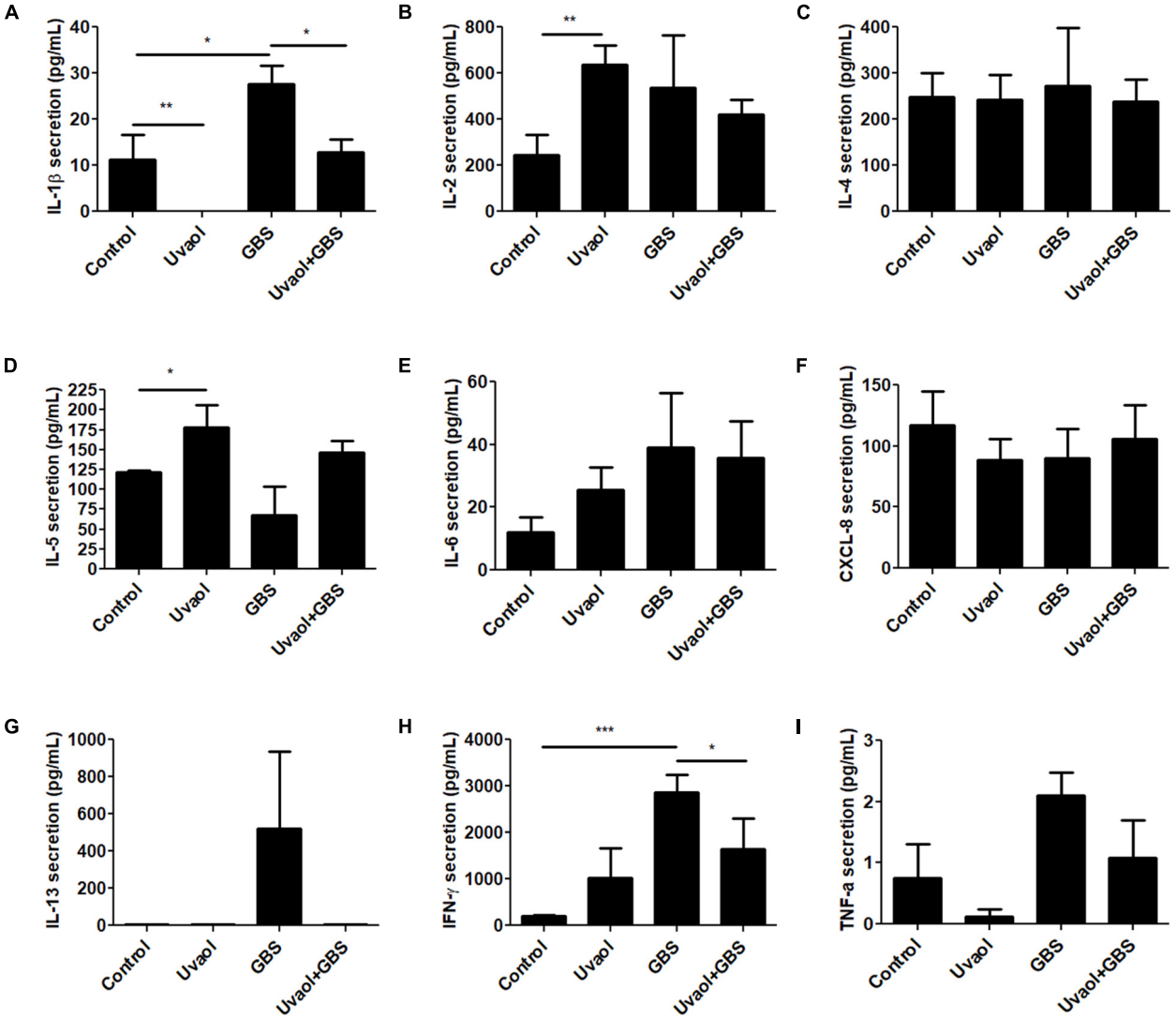

FIGURE 3 | Cytokine production of trophoblast cells is altered by GBS and uvaol. HTR-8/SVneo cells were treated with 10-μM uvaol (uvaol), GBS at 1×10^6 CFU, or both for 24 h, and the supernatants were analyzed for IL-1β **(A)**, IL-2 **(B)**, IL-4 **(C)**, IL-5 **(D)**, IL-6 **(E)**, CXCL-8 **(F)**, IL-13 **(G)**, IFN-γ **(H)**, and TNF-α **(I)** by flow cytometry. Bar graphs represent mean values ± S.E.M.; $n = 6$ in duplicate. *, $p < 0.05$; **; $p < 0.01$; ***, $p < 0.001$. One-way ANOVA with Tukey *post hoc* test.

secretion was 217.7 ± 5.62 pg/ml, CXCL-8 secretion was 1,038 ± 149.6 pg/ml, EGF secretion was 2.37 ± 0.55 pg/ml, FGF-β secretion was 16.09 ± 2.78 pg/ml, IL-6 secretion was 379.6 ± 32.92 pg/ml, PlGF secretion was 251.1 ± 60.25 pg/ml, sPECAM-1 secretion was 1,408 ± 182.7 pg/ml, TNF-α secretion was.36 ± 0.36 pg/ml, and VEGF secretion was 1,244 ± 137.4 pg/ml (**Figures 6B–J**). Cocultured of trophoblast cells with endothelial cells (Control group) changed their basal production. As such, Angiopoietin-2 secretion was increased to 308.5 ± 4.33 pg/ml ($p < 0.05$), CXCL-8 was increased to 1,874 ± 360.7 pg/ml ($p < 0.05$), and VEGF was also increased to 1,915 ± 417.6 pg/ml ($p < 0.05$) (**Figures 6B,C,J**). Inversely, trophoblast cells addition reduced IL-6 and sPECAM-1 secretion by endothelial cells. IL-6 secretion was reduced to 260.3 ± 16.38 pg/ml ($p < 0.05$), and sPECAM-1 was remarkably reduced to 373.3 ± 293.9 pg/ml ($p < 0.001$) (**Figures 6F,H**). Trophoblast cells cultured under the same conditions but, without endothelial cells, did not produce detectable levels of Angiopoietin-1, while no molecules were solely changed by uvaol, and GBS incubation increased IL-6 production from 465.1 ± 40.76 to 846.3 ± 32.74 pg/ml ($p < 0.05$) (**Supplementary Figure 2**).

Uvaol Prevented the Group B *Streptococcus*-Induced Increase of CXCL-8 and IL-6 in the Three-Dimensional Vascular Invasion Coculture Model

Group B *Streptococcus* (GBS) at 10^6 CFU induced the production of inflammatory cytokines that also have vasoactive functions,

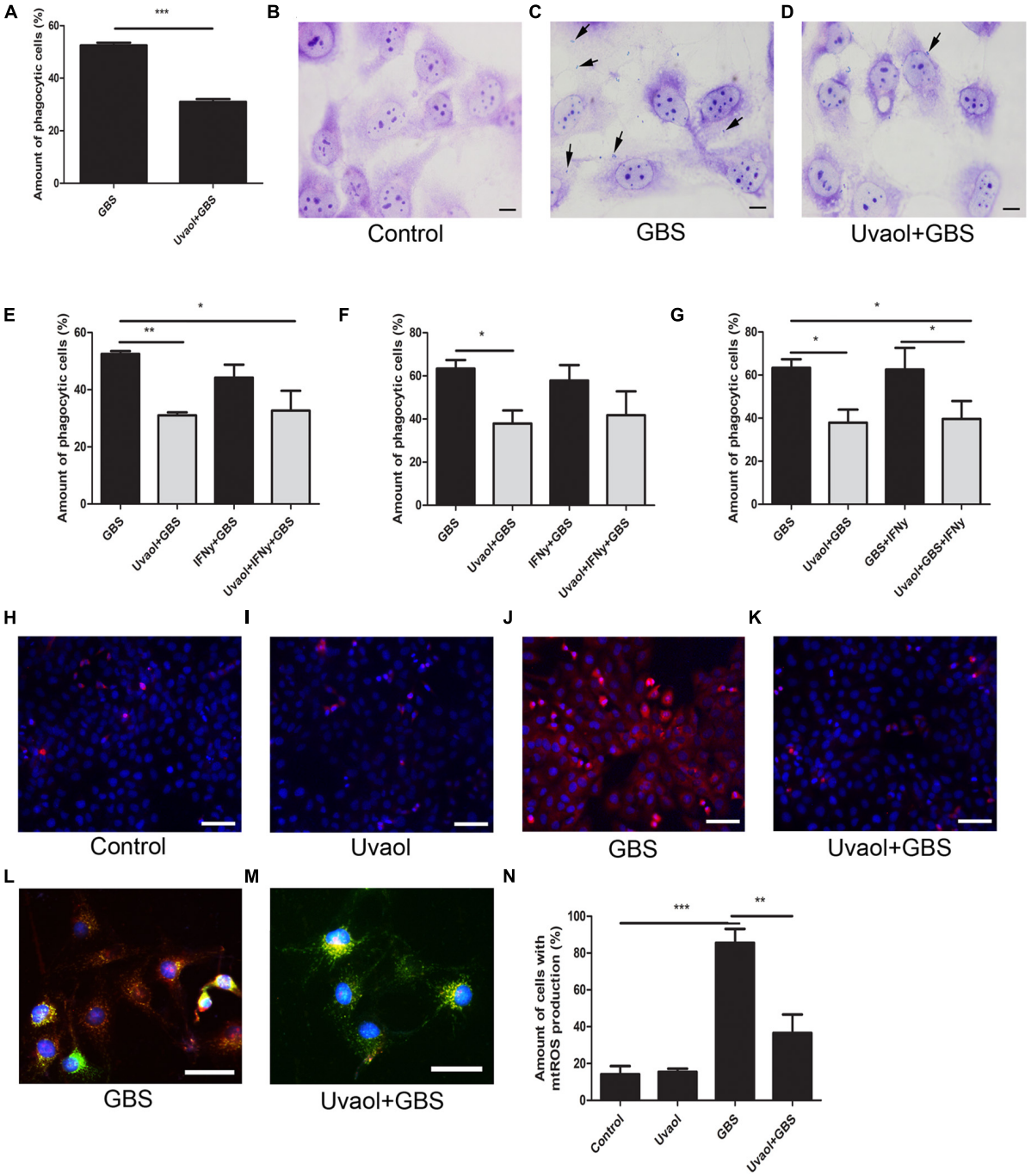

FIGURE 4 | Uvaol reduces trophoblast cells phagocytosis and mtROS production. **(A)** Phagocytosis quantification after 2 h of GBS inoculation. HTR8SV/neo cells were treated with 10-μM uvaol for 1 h and incubated with GBS at 10^6 CFU for further 2 h. A bar graph represents mean values ± S.E.M.; $n = 4$ in triplicate. ***; $p = 0.004$. Paired *t-test*. **(B–D)** Cells were stained by Giemsa staining for phagocytosis assessment, where: **(B)** control, **(C)** GBS, and **(D)** uvaol + GBS group. Black arrows show bacteria in trophoblast cells. 1,000 × magnification. Scale bars represent 20 μm. **(E)** Phagocytosis quantification after 2 h of GBS inoculation. HTR8SV/neo cells were treated for 24 h with 100 UI/ml IFN-γ and/or 1 h with 10-μM uvaol before GBS at 10^6 CFU incubation for further 2 h. **(F)** Phagocytosis quantification after 24 h of GBS inoculation. HTR8SV/neo cells were treated for 24 h with 100-UI/ml IFN-γ and/or 1 h with 10-μM uvaol before GBS at 10^6 CFU incubation for further 2 h. **(G)** Phagocytosis quantification after 24 h of GBS inoculation. HTR8SV/neo cells were treated for 1 h with 10-μM uvaol before GBS at 10^6 CFU for 2 h, and 100 UI/ml IFN-γ for further 22 h. A bar graph represents mean values ± S.E.M.; $n = 3$ in triplicate. *; $p < 0.05$, **; $p < 0.01$. One-way ANOVA with Tukey *post hoc* test. **(H–K)** Cells were stained with MitoSOX red and nuclei with DAP-I, where: **(H)** control, **(I)** uvaol, **(J)** GBS, and **(K)** uvaol + GBS group. 200× magnification. Scale bars represent 200 μm. **(L,M)** Cells were stained with MitoTRACKER green, MitoSOX red, and nuclei with DAP-I were **(L)** GBS and **(M)** uvaol + GBS group. 1,000× magnification. Scale bars represent 100 μm. **(N)** mtROS quantification. A bar graph represents mean values ± S.E.M.; $n = 3$ in triplicate. **; $p < 0.01$, ***; $p < 0.001$. One-way ANOVA with Tukey *post hoc* test.

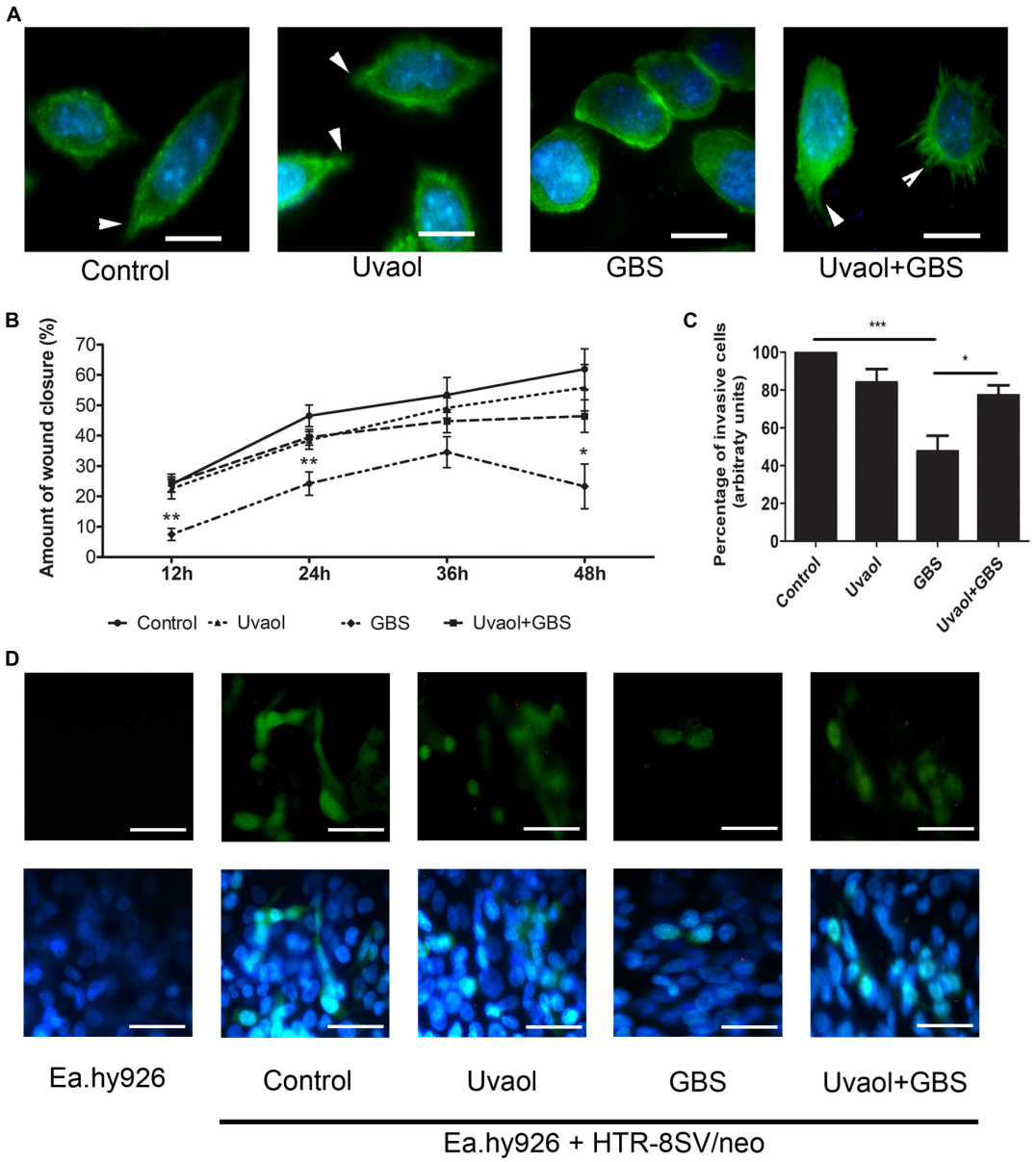

FIGURE 5 | GBS reduces trophoblast cell motility, and uvaol prevents reduction. **(A)** Phalloidin (green) and nuclei staining (DAP-I) of trophoblast cells from control, 10-μM uvaol, GBS at 1×10^6 CFU, or both (uvaol + GBS). Filopodia are identified by white arrowheads. 1,000× magnification. Scale bars represent 50 μm. **(B)** Percentage of *in vitro* wound closure per time point analyzed (12, 24, 36, and 48 h). A bar graph represents mean values ± S.E.M.; *, $p < 0.05$; **; $p < 0.01$. Two-way ANOVA with Bonferroni *post hoc* test. **(C)** Percentage of invasive trophoblast cells through 3D vascular invasion assay. A bar graph represents mean values ± S.E.M.; *, $p < 0.05$; ***; $p < 0.001$. One-way ANOVA with Tukey *post hoc* test. **(D)** Representative images from the 3D coculture vascular invasion assay with CFSE-stained trophoblast cells and nuclei stained with DAP-I, where: (first column) Ea.hy926 endothelial cells, (second column) control (endothelial + trophoblast cells), (third column) with uvaol, (fourth column) with GBS, (fifth column) with uvaol and GBS. 400× magnification. Scale bars represent 200 μm. All experiments were $n = 3$ in triplicate.

such as CXCL-8 and IL-6, whereas uvaol addition completely prevented their increase. In fact, CXCL-8 secretion dramatically increased to 3,285 ± 383.5 pg/ml ($p < 0.05$, compared to Control), whereas the uvaol + GBS group had 2,372 ± 179.8 pg/ml ($p < 0.05$, compared to GBS) (**Figure 6C**). The same pattern occurred to IL-6, which was upregulated to 390.2 ± 49.85 pg/ml in the GBS group ($p < 0.05$, compared to Control), and reduced to 254.7 ± 24.12 pg/ml in the uvaol + GBS group ($p < 0.05$, compared to GBS) (**Figure 6F**). It is worth noting that trophoblast cells 2, dimensionally cultured alone with GBS, had no increase in CXCL-8 and IL-6 levels (**Figures 4E,F**), whereas the results with three-dimensional culture had an increase in IL-6 (**Supplementary Figure 2**). Although we cannot exactly affirm from which cell CXCL-8 and IL-6 increased production

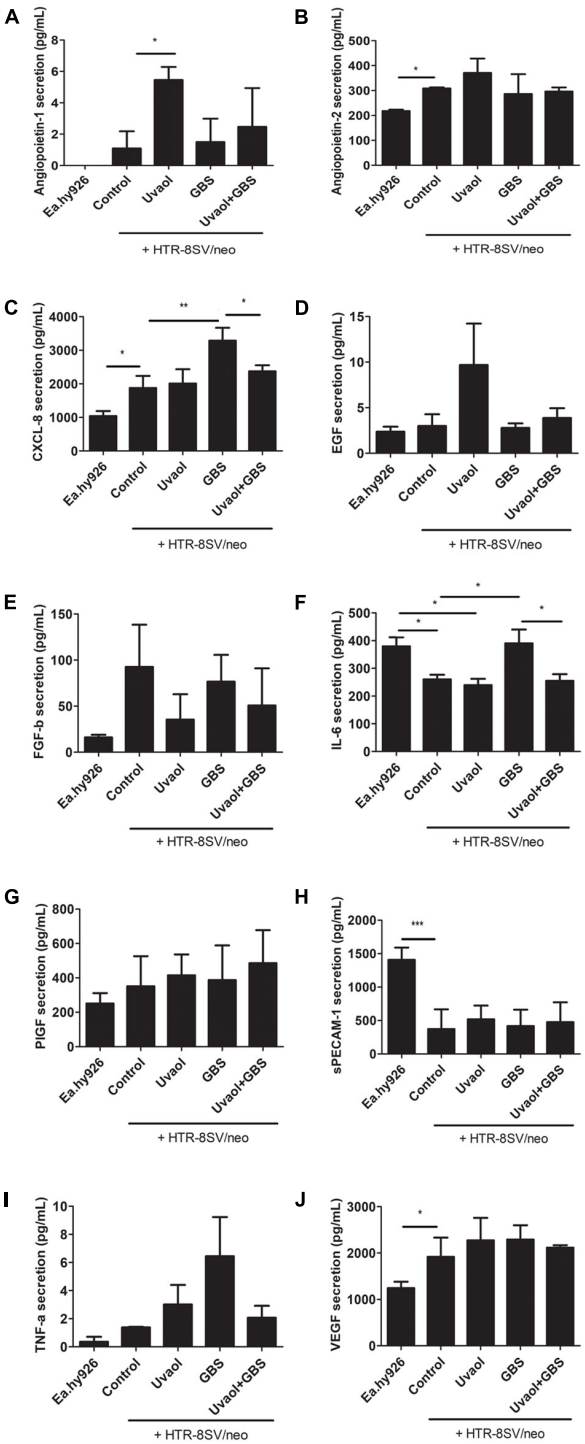

FIGURE 6 | Vasoactive factors secretion from the 3D coculture vascular invasion assay. The bottom supernatants from the 3D coculture vascular invasion assay were collected and analyzed for angiopoietin-1 **(A)**, angiopoietin-2 **(B)**, CXCL-8 **(C)**, EGF **(D)**, FGF-b **(E)**, IL-6 **(F)**, PlGF **(G)**, sPECAM-1 **(H)**, TNF-α **(I)**, and VEGF **(J)** through flow cytometry. The analyzed groups were composed by Ea.hy926 endothelial cells alone, with HTR8SV/neo trophoblast cells (control), added with 10-μM uvaol or GBS at 1×10^6 CFU, or both (uvaol + GBS). A bar graphs represent mean values ± S.E.M.; $n = 3$ in triplicate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

was derived, the 3D co-culture model was a powerful method that increased the analysis complexity and provided CXCL-8 and IL-6 as key molecules in the interaction of trophoblast cells, endothelial cells, and GBS.

Uvaol Prevented the Group B *Streptococcus*-Induced Increase of CXCL-8 and IL-6 in Placental Explants Exposed to Group B *Streptococcus*

Placental chorionic villi explants were also analyzed for their production of vasoactive factors since their production can affect maternal-fetal blood vessels. As a result, EGF was not found in any analyzed group. Angiopoietin-1 tended to increase its production in the GBS group (**Figure 7A**), whereas Angiopoietin-2, FGF-b, sPECAM-1, and TNF-α had unchanged levels despite of the addition of GBS and/or uvaol (**Figures 7B–E**). Uvaol alone increased VEGF production compared to control (4.989 ± 3.06 pg/ml to 23.03 ± 4.8 pg/ml, $p < 0.01$), although, when with the GBS, the effect disappeared (**Figure 7F**). Interestingly, as the same patterns observed in our 3D vascular invasion coculture model, GBS greatly increased IL-6 production compared to control (13,800 ± 9,039 pg/ml to 194,000 ± 48,490 pg/ml, $p < 0.01$) (**Figure 7G**), the same occurring to CXCL-8 (3,706 ± 1,428 pg/ml to 9,220 ± 754.8 pg/ml, $p < 0.05$) (**Figure 7H**). As such, uvaol addition prevented the GBS-induced increase in IL-6 and CXCL-8, as IL-6 levels reduced to 25,580 ± 11,680 pg/ml ($p < 0.01$) (**Figure 7G**), and CXCL-8 levels reduced to 2,740 ± 719.8 pg/ml ($p < 0.05$) (**Figure 7H**).

DISCUSSION

Herein, we showed that incubation of inactivated GBS at 106 CFU on HTR-8/SVneo trophoblast cells and term chorionic villi explants have not induced changes in their viability; therefore, it was used in our *in vitro* experiments. Nevertheless, GBS incubation, either live or inactivated, is known to induce cell death in trophoblast cells (Kaplan et al., 2008; Botelho et al., 2019). Since live GBS can induce maternal death, *in vitro* studies have certain limitations of reproducibility, such as the maternal health situation, trimester of GBS infection, presence of chorioamnionitis, and its degree. As such, live GBS can lead to wide variance of clinical outcomes that are difficult to reproduce *in vitro*. To overcome such issues, we employed inactivated GBS in this study, where we could analyze the specific effects caused only by the physical contact of the bacteria to trophoblast cells, isolating infection variables. Corroborating our rationale, previous studies already showed similar results when live and inactivated GBS were tested in animal models, where placental, fetal, and offspring outcomes (chorioamnionitis, FIRS, pathological, and behavioral effects on the offspring) were alike (Allard et al., 2016, 2019; Giraud et al., 2020).

Our results with a non-lethal inactivated GBS concentration highlight the need for further studies on GBS effects on the placenta and its cells. Trophoblast cells had intense alterations in their biochemical signature, observed in Raman spectroscopy

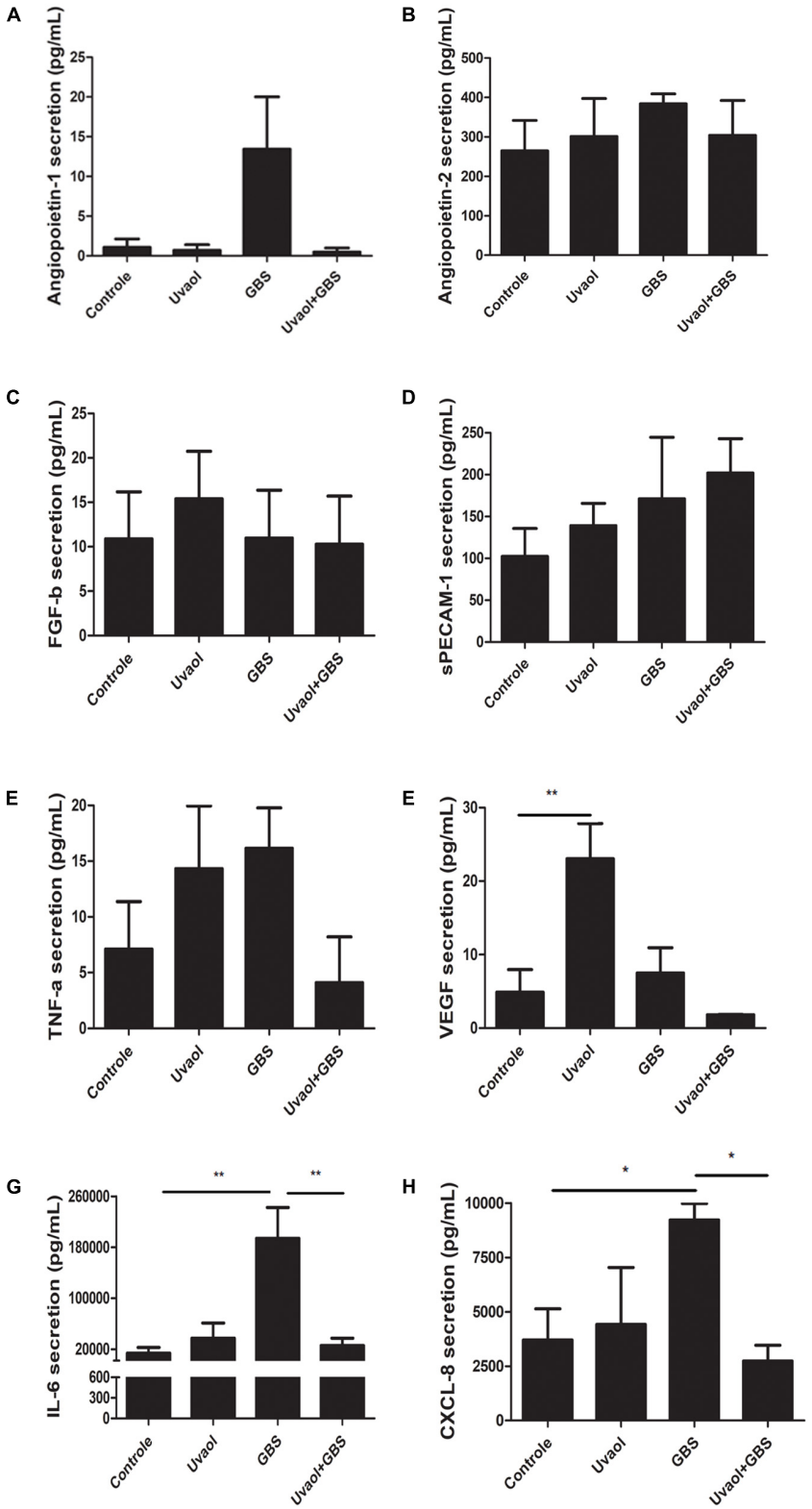

FIGURE 7 | Vasoactive factors secretion by term chorionic villi explants. The supernatants from the term chorionic villi explants were collected and analyzed for angiopoietin-1 **(A)**, angiopoietin-2 **(B)**, FGF-b **(C)**, sPECAM-1 **(D)**, TNF-α **(E)**, VEGF **(F)**, IL-6 **(G)**, and CXCL-8 **(H)** through flow cytometry. The analyzed groups were composed by control, added with 50-μM uvaol or GBS at 1×10^6 CFU, or both (uvaol + GBS). A bar graphs represent mean values ± S.E.M.; $n = 5$ in duplicate. *, $p < 0.05$; **, $p < 0.01$.

multivariate analysis using different models (PCA, HCA, and OPLS-DA). All the different analyses demonstrated their potential by separating cells into four distinct clusters that were equivalent to the groups being studied. The OPLS-DA model also found 86% of predictive accuracy on samples distinctively separated among the groups. It is important to highlight that this study is the first to use Raman spectroscopy to show the biochemical differences caused by GBS in trophoblast cells. In one of the few publications regarding Raman spectroscopy in the placenta, Chen et al. (2014) found some band intensities to be changed in preeclamptic placentas compared to healthy placentas. In their study, preeclamptic placentas had differences in bands related to amino acids and amide I, assigning the amide I difference to protein structural disorders. Also, they found unusual spectra for phenylalanine and tryptophan, which correlated with oxidative modifications common in preeclampsia (Chen et al., 2014). Interestingly, we found some similar modifications in our trophoblast cells incubated with GBS, which had altered levels of bands related to phenylalanine and amide I. Other bands related to lipids or protein conformations (amide II and amide III) were also deeply changed. For example, the reduction of 1,662 cm^{-1} intensity has been related to decreased proliferation and migration due to protein abnormalities (Wesełucha-Birczyńska et al., 2013). We also identified altered bands related to DNA, RNA, cholesterols, fatty acids, and membrane phospholipids, which could indicate changes in membrane assembly, stiffness, activity, and function (Gelder et al., 2007; Movasaghi et al., 2007; Czamara et al., 2015). The uvaol treatment before GBS incubation halved the number of bands altered by GBS only. These bands were mainly related to DNA, RNA, phenylalanine, glutathione, glycosaminoglycans, myristic acid, membrane phospholipids, cholesterols, and amide I, whereas uvaol did not affect other bands related to amide I, amide III, fatty acids, lipids, proteins, and purines. Further studies need to be performed to deeply investigate the molecular interactions and effects provoked by uvaol. Nonetheless, the antagonist effect observed on DNA and RNA-related bands was sound, protecting cells from small alterations that could lead to damage/death soon. This protection might be connected with a possible protective effect on the cytoplasmic membrane, observed by membrane phospholipids, cholesterols, glycosaminoglycans, and myristic acid-related bands. Interestingly, our previous study showed that GBS at 10^8 CFU disrupted the cytoplasmic membrane, decreased its stiffness, and induced trophoblast cell death (Botelho et al., 2019). As such, it seems that the GBS-inducing alterations are still present here, but not in intensity to induce cell death at the time point we analyzed.

Regarding cytokines production, IL-1β and IFN-γ were increased by GBS at 10^6 CFU, similarly to the published alterations provoked by 10^8 CFU (Botelho et al., 2019), although IL-2 and IL-4 were not changed herein. IL-1β is a central cytokine produced in GBS response (Bergeron et al., 2013; Gupta et al., 2014), and IFN-γ is also secreted by activated CD4 + T cells in mice models of GBS infection (Chen et al., 2015). Both cytokines are also closely related to vascular dysfunctions, considered predictive and prognostic markers of vascular diseases (LaMarca et al., 2011; Raghupathy, 2013). These cytokines could be produced by indirect binding of inactive GBS to trophoblast cell membranes and by phagocytosis. The phagocytosis was accessed for live and inactive GBS, and live GBS was less phagocyted than inactive GBS, which could be possible due to evasion mechanisms of live GBS (Patras and Nizet, 2018). The GBS phagocytosis mechanisms vary widely due to 10 known capsular serotypes, and, to our knowledge, no specific mechanisms were reported to each one of these serotypes, as phagocytosis in macrophages strongly differed across clinical GBS isolates, but no correlation was found to capsular serotypes, genetic sequence types, pilus types or clinical sources (Rogers et al., 2018). Surprisingly, recombinant IFN-γ was not able to increase or restore phagocytosis in our model, as previously reported by others (Albieri et al., 2005; Tachibana et al., 2015). Regarding uvaol, it is not described in the literature to reduce phagocytosis, although a possible explanation would be the effects visualized in Raman spectroscopy related to the cell membrane and/or by membrane stiffness increase induced by this triterpene (Botelho et al., 2019).

Moreover, cells increased the production of mtROS. In several biological systems, mtROS are thought to play key roles in the adaptation to different stimuli, including hypoxia, cytokine stimulation, and calcium influx (Sena and Chandel, 2012). Although the redox biology of pregnancy and associated complications remain largely unexplored, evidence suggests that dysregulation of mtROS homeostasis causes mitochondrial dysfunction and oxidative stress, and these events are associated with the onset of adverse gynecological outcomes, mostly linked to vascular diseases (Sánchez-Aranguren et al., 2014; Karaa et al., 2019). As such, the one proposed mechanism of endothelial dysfunction is that poor trophoblast invasion is caused by increased ischemia/reperfusion and inflammation, which trigger oxidative stress from unbalanced ROS formation, generating superoxide and peroxynitrite. Both are thought to induce lipid peroxidation, tyrosine nitration, protein modifications, and DNA damage, leading to endothelial dysfunction (Sánchez-Aranguren et al., 2014). Interestingly as well, these modifications caused by superoxide production correlate with several altered bands in Raman spectroscopy results, and further studies need to be performed to better understand these correlations. An important finding was the protective effect of uvaol on preventing mtROS formation in our model. Corroborating our results, other studies also showed antioxidant effects of uvaol treatment (Allouche et al., 2010; Bonel-Pérez et al., 2020).

Additionally, we showed that trophoblast cells incubated with GBS had a reduction of filopodia and that the F-actin cytoskeleton was slightly disorganized. Cell motility was impaired as well, as, in the GBS group, cells closed the gap from the *in vitro* wound-healing assay three times slower than in control. Interestingly, trophoblast-reduced migration is one of the key events in vascular dysfunction and early-onset preeclampsia establishment (Fisher, 2015; Barrientos et al., 2017). Under physiological conditions, extravillous cytotrophoblast cells invade and remodel uterine spiral arteries, replacing endothelial cells and muscular linings of the uterine spiral arterioles and arteries. As a result, the spiral arteries diameter increases, allowing enhanced perfusion and increased metabolic uptake by the maternal-fetal interface. However, when the invasion is shallow, the mean arterial

diameter stays approximately half of that of remodeled arteries from normal placentas (Fisher, 2015; Barrientos et al., 2017). Therefore, spiral arteries transform into high resistance vessels with endothelial cells not being entirely replaced by trophoblast cells, which activates immune cells and leads to vascular dysfunction (Kell and Kenny, 2016). Additionally, we used a 3D vascular invasion co-culture model where trophoblast cells could invade through endothelial cells to confirm and expand the motility impairment. The results depicted that the trophoblast invasion was remarkably affected by GBS. Other studies have also described the possibility of subclinical placental infections, increasing inflammation and ROS production, and decreasing trophoblast migration and endothelial invasion (López-Jaramillo et al., 2008; Kell and Kenny, 2016). Hence, uvaol treatment prevented migration and invasion impairment induced by GBS incubation. Recently, a study has corroborated our results, with uvaol presenting *in vivo* increase in wound healing and *in vitro*, endothelial cells and fibroblasts had their migration upregulated by uvaol treatment, which described to depend on PKA and p38/MAPK-signaling pathways in endothelial cells (Carmo et al., 2020).

To analyze if GBS could, indeed, provoke endothelial dysfunction, we analyzed the supernatants from placental chorionic villi explants exposed to GBS and from the bottom supernatants from the 3D co-culture invasion assay. Both models had the same vasoactive factors being increased by GBS: IL-6 and CXCL-8. Although IL-6 regulation of trophoblast cell migration is still debated (Champion et al., 2012; Sokolov et al., 2015), its roles in vascular dysfunction are well-established. IL-6 has increased levels in preeclamptic patients (Lockwood et al., 2008) and promotes thrombogenesis, increased expression of endothelial adhesion molecules, and vascular permeability (Roldán et al., 2003), increased vascular fibrosis, immune cell recruitment, endothelial activation, and its further dysfunction (Didion, 2017). Regarding CXCL-8, it is also increased in preeclamptic patients (Valencia-Ortega et al., 2019), and this potent neutrophil chemoattractant increases vascular permeability (Petreaca et al., 2007). It can help neutrophils infiltrate uterine spiral arteries and release ROS, MMPs, and thromboxane, increasing inflammation and presenting a pivotal role in vascular dysfunction (Pinheiro et al., 2013). Moreover, Pinheiro and collaborators (Pinheiro et al., 2013) found that increased CXCL-8 positively correlated with increased IFN-γ in preeclampsia. In this context, IFN-γ decreases trophoblast migration and placental villi outgrowths (Lash et al., 2006; Verma et al., 2018) and upregulates IL-6 and CXCL-8 (Wissen et al., 2002), involved in vascular dysfunction (LaMarca et al., 2011; Pinheiro et al., 2013; Raghupathy, 2013).

Due to the severity of GBS infection, Europe and United States recommend intrapartum antimicrobial prophylaxis based on a universal intrapartum GBS screening strategy (di Renzo et al., 2015). Although prophylaxis with antibiotics is proven effective (Vornhagen et al., 2017), their use has been associated with an increased risk of neonatal death, with a risk ratio of 1.57 (Flenady et al., 2013). Also, ampicillin treatment of GBS infection in a rat model of chorioamnionitis increased IL-1β and polymorphonuclear infiltration, which are thought to affect fetal neurodevelopment (Giraud et al., 2020). Furthermore, GBS has also proved to become resistant to several antibiotics, such as tetracycline, erythromycin, and clindamycin (Nascimento et al., 2019), which add to the need for preventive strategies. One possibility is the use of nutraceuticals, such as vitamin D, folic acid, and resveratrol, which are thought to prevent the development of pregnancy-related hypertensive disorders (Schütz et al., 2017; Fogacci et al., 2020). As such, we believe that uvaol could be a potential candidate, and further studies need to be conducted to deepen our knowledge of uvaol signaling mechanisms and *in vivo* studies as well since we understand the limitations of our study and the need to be cautious about the translation of *in vitro* to *in vivo* or clinical studies.

Furthermore, our study enhances the need for GBS tracking during pregnancy, suggesting even earlier tracking than the current recommendations, since lower concentrations of GBS that could infect women subclinically might already cause trophoblast impairment, inflammation, and vascular dysfunction. Additionally, the possibility to improve maternal and neonatal pregnancy outcomes with olive oil-derived dietary supplementation deserves attention, as diets enriched by olive oil consumption are already linked to fewer cases of preeclampsia and prematurity (Laws et al., 2009; Shen et al., 2015; Irianti et al., 2017). Therefore, it is important to highlight the salutary effects of uvaol treatment to trophoblast cells, as it seems to be a potent nutraceutical capable of avoiding deleterious effects linked to GBS exposure, presenting important anti-inflammatory, antioxidant, and pro-motility effects.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The study was approved at the Federal University of Alagoas Ethics Committee, also approved by Brazilian National Ethical Committee System – Plataforma Brasil (52237915.5.0000.5013). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ALMS was responsible for maintaining the culture, uvaol, and GBS treatments, the *in vitro* wound-healing assay, and the 3D co-culture invasion assay. ECOS was responsible for Raman spectroscopy, spectrum analysis, and PCA and HCA analysis. RMB was responsible for MTT and Annexin V/PI staining. LPGT helped with *in vitro* assays and Raman spectroscopy. ALXM helped in the individual Raman intensity peak analysis and phalloidin staining. IBACR was responsible for placental explants culture and experiments. LIMA was responsible for phagocytosis assay and helped with flow cytometry assays. AKAS were responsible for mtROS assay and the phagocytosis assay with

IFN-γ. KSNP helped in cell culture, ELISA, and overall analysis. ISBT helped with live GBS culture and experiments and figures preparation. M-JA was responsible for GBS culture, titration, inactivation, and preparation to send it to Brazil. GS provided invaluable help in review and editing the manuscript. STS was responsible for Raman spectral data supervision, validation, and statistical analysis. EJSF helped in reviewing and editing this manuscript, as well as supervision of Raman spectroscopy data. KSCB was responsible for all LegendPLEX assays, including analysis and discussion in the manuscript. AUB was responsible for organizing results and data analysis, writing the original draft, and general supervision. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.766382/full#supplementary-material

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Assessing the Role of Uric Acid as a Predictor of Preeclampsia

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We assessed the diagnostic utility of uric acid for the prediction of preeclampsia. An observational prospective approach was carried out during 2014. Preeclamptic women were classified into 4 groups accordingly to the onset of preeclampsia and the presence of intrauterine growth restriction (IUGR). Serum uric acid levels, urea, and creatinine were measured. Receiver operating curves (ROC) of the uric acid levels ratio (UAr) between a dosage before and after the 20th week of gestation were performed. One thousand two hundred and ninety-third pregnant women were enrolled in this study. Eight hundred ten had non-complicated pregnancies, 40 preeclampsia, 33 gestational hypertension, and 20 IUGR without preeclampsia. Uric acid significantly raised after 20 weeks of gestation in women who develop preeclampsia before 34 weeks (Group A) or in those who develop preeclampsia after 37 weeks associated with IUGR (Group C). In women who develop preeclampsia after 34 weeks without IUGR (Groups B and D), uric acid increased after the 30th week of gestation. In all groups, UAr was greater than 1.5. In gestational hypertension, UAr was superior to 1.5 toward the end of gestation, while in IUGR without preeclampsia, the behavior of serum uric acid was similar to non-complicated pregnancies. In all cases, urea and creatinine showed normal values, confirming that patients had no renal compromise. ROC area was 0.918 [95% confidence interval (CI): 0.858–0.979) for the preeclampsia group and 0.955 (95% CI: 0.908–1.000) for Group A. UAr at a cut-off point ≥1.5 had a very low positive predictive value, but a high negative predictive value of 99.5% for preeclampsia and it reached 100% for Group A. Thus, a UAr less than 1.5 may be a helpful parameter with a strong exclusion value and high sensitivity for those women who are not expected to develop preeclampsia. Additionally, this low-cost test would allow for better use of resources in developing countries.

Keywords: uric acid, diagnostic value, biomarker, preeclampsia, intrauterine growth restriction (IUGR)

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INTRODUCTION

Preeclampsia is a multisystem disorder of unknown etiology that is unique to human pregnancy. Its clinical presentation is highly variable, and it complicates 10 million pregnancies annually, resulting in 76,000 maternal and 500,000 fetal or newborn deaths worldwide. In Latin America, its incidence is between 5 and 7% and it is the leading cause of maternal death (∼26%) while in Argentina,

hypertensive disorders of pregnancy are responsible for 12.4% of maternal deaths (Abalos et al., 2013; Giachini et al., 2017; Guevel, 2018).

The consequences of this syndrome are not limited to pregnancy. They can lead to permanent vascular and metabolic damage and an increased risk of developing cardiovascular diseases in both the mother and the offspring (Huda and Greer, 2011; Abalos et al., 2014; Giachini et al., 2017). Although this pathology cannot be cured its early detection and referral to a high-complexity center are crucial to minimizing pregnancy complications and the long-term consequences. However, up to now, women who develop preeclampsia are only diagnosed after the onset of clinical symptoms, which makes patient management much more complicated.

Because of the serious consequences of this syndrome, it is still a challenge to predict which women are at risk of developing preeclampsia. So far, several promising biomarkers that could be used to make an early diagnosis have been identified. Anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), as well as pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) have demonstrated some benefit in the prediction or diagnosis of preeclampsia, as well as in the understanding of its etiology (Huppertz, 2018; McCarthy et al., 2018; Baert et al., 2021; Dröge et al., 2021; Lim et al., 2021). However, the choice of a biomarker should not only be related to its biological characteristics and its participation in the pathogenesis of the disease, but also to the feasibility of being used as a screening tool. A marker that requires complex technology and does not show a positive cost-benefit would be inapplicable. This could be even more relevant in low-income countries, where the number of deaths related to hypertensive pregnancies is higher than in developed countries (Giachini et al., 2017).

Although an association between elevated serum uric acid levels and preeclampsia has been known since the beginning of the 20th century and although this increase is considered, by many authors, as a marker of the severity of the disease, the clinical utility of this knowledge is still debated (Slemons and Bogert, 1917; Corominas et al., 2014; Pecoraro and Trenti, 2020; Zhao et al., 2021).

In uncomplicated pregnancies, serum uric acid concentrations decrease 25–35% due to the pregnancy-induced expansion of the blood volume, the increase in renal blood flow, the glomerular filtration rate, and the uricosuric action of estrogen (Nicholls et al., 1973; Lind et al., 1984; Carter and Child, 1989; Martínez-Gascón et al., 2016). Later in pregnancy, the serum uric acid levels rise until the end of pregnancy, as a consequence of the increase in fetal production, the decreased binding to albumin, and the decline in renal clearance (Lind et al., 1984; Nwagha et al., 2009; Amini et al., 2014).

In preeclamptic pregnancies, the increase in serum uric acid levels may be related to decreased uric acid excretion (Roberts et al., 2005; Khaliq et al., 2018). However, the uricemia increase precedes the proteinuria increase (Corominas et al., 2014). More recently, the increased oxidative stress and the formation of reactive oxygen species were proposed as other contributing sources of the hyperuricemia observed in preeclampsia (Many et al., 1996; Bainbridge and Roberts, 2008; Masoura et al., 2015).

On the other hand, due to the uric acid interaction with proinflammatory cytokines, increased levels of uric acid in the plasma of patients with preeclampsia may indicate a direct contribution to the pathophysiology of this syndrome by its ability to promote inflammation (Mulla et al., 2011; Matias et al., 2015; Shirasuna et al., 2020).

However, the clinical significance of serum uric acid concentrations in monitoring hypertension in pregnancy is either minimally predictive or not predictive. A possible explanation is that the dosages were generally carried out at the beginning of gestation or after the onset of the clinical manifestations (Thangaratinam et al., 2006; Bellomo et al., 2011; Corominas et al., 2014; Chen et al., 2016; Pecoraro and Trenti, 2020).

Previously, we found that uric acid levels in preeclamptic pregnant women increased by at least 1.5 times after the 20th week of gestation, with no changes in uremia or creatininemia, showing the absence of renal compromise. We proposed that a Uric acid ratio (UAr) greater than 1.5 may be related to preeclampsia (Corominas et al., 2014). However, its predictive value is still under discussion.

Here, we propose to study the uric acid behavior throughout gestation to evaluate its predictive value to define the risk of developing preeclampsia.

MATERIALS AND METHODS

Subjects

An observational prospective study was conducted to examine the behavior of serum uric acid levels throughout pregnancy and to ascertain its predictive value in determining the risk of developing preeclampsia. The study was approved by the Institutional Review Board and written consent was obtained from all subjects.

This study was carried out between January to December 2014, at the "Hospital Nacional Profesor Alejandro Posadas," Buenos Aires, Argentina.

During this period, 1,293 pregnant women who received full antenatal care at the hospital were enrolled in this study. All women belonged to the white Hispanic ethnic group.

Previously, we have reported that uric acid levels did not change before the 20th week of gestation in women who developed preeclampsia, while in the second half of pregnancy these levels abruptly increased (Corominas et al., 2014). Consequently, we divided pregnancy into 4 stages to analyze the uric acid increase throughout pregnancy:

Stage 1: before 20 weeks of gestation

Stage 2: between 20 and 30 weeks of gestation

Stage 3: between 31 and 34 weeks of gestation

Stage 4: after 35 weeks of gestation.

In addition, preeclamptic pregnant women were stratified into 4 groups according to the onset of preeclampsia and the presence of low birth weights (Myatt et al., 2014):

Group A: women who develop preeclampsia before 34 weeks

Group B: women who develop preeclampsia between 34 and 36.9 weeks

Group C: women who develop preeclampsia after 37 weeks with fetal growth restriction

Group D: women who develop preeclampsia after 37 weeks without low birth weight.

Serum samples from each pregnant woman were collected throughout pregnancy and stored at −80°C until analyzed.

The gestational age of the patients at the time of collection was calculated as the time between the first day of the last menstrual period and the date of the blood analysis.

Uncomplicated gestations were defined as healthy pregnancies, with no underlying maternal condition that could adversely affect the pregnancy.

Gestational hypertension and preeclampsia were defined based on the FLASOG (Federacion Latinoamericana de Sociedades de Obstetricia y Ginecología) guidelines.

Gestational hypertension was defined as diastolic blood pressure persistently ≥140 mmHg and/or ≥90 mmHg diastolic, on two occasions (at least 6 h apart), after the 20th week of gestation without proteinuria, in a previously normotensive woman.

Preeclampsia was defined as systolic blood pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg, with proteinuria ≥0.3 g/day or 2 pluses on urine dipstick after the 20th week of gestation in a previously normotensive patient.

Intrauterine growth restriction (IUGR) was defined as a fetal birth weight lower than the 10th percentile for the gestational age. Gestational age-specific birth weight centile was based on data provided by WHO[1].

Women who carried on multiple pregnancies, women with a diagnosis of chronic kidney disease, chronic hypertension, liver disease, collagen vascular disease, diabetes, major fetal abnormalities, cardiovascular disease, cancer, and those who declined to take part in the study were all excluded.

Serum Uric Acid Quantification

Serum uric acid was measured using a color-enzymatic diagnostic kit by Roche (Buenos Aires, Argentina) in a Hitachi 917 analyzer according to the manufacturer's protocol. Reference values for women are 2.4–5.7 mg/dL.

[1]http://www.who.int/childgrowth/mgrs/en/

Serum Urea and Creatinine Quantification

Serum urea was measured by an enzymatic diagnostic kit supplied by Roche (Buenos Aires, Argentina) in a Hitachi 917 analyzer according to the manufacturer's protocol. Reference values are 0.10–0.50 mg/dL.

Serum creatinine was assessed by a colorimetric-kinetic kit based on the Jaffe reaction supplied by Roche (Buenos Aires, Argentina) in a Hitachi 917 analyzer according to the manufacturer's protocol. The reference normal values are 0.5–0.9 mg/dL.

Pre-pregnancy Body Mass Index

BMI [weight (kg)/height $(m)^2$] was based on measured height and maternal report of pre-pregnancy weight.

Statistical Analysis

We analyzed the data with two sample *T*-tests for equal variances, and with Wilcoxon signed-rank test when data were not normally distributed.

Multiple comparisons were evaluated by one-way analysis of variance (ANOVA test) followed by Fisher LSD test.

Association between UAr and BMI, UAr and parity, and UAr and maternal age were checked by chi-square test. Phi coefficient, Cramer's V, and the contingency coefficient (Karl Pearson) were used to investigate the degree of association, excluding the effect of sample size. Correlation coefficients from 0 to 0.25 indicate little dependency and higher than 0.6 show great dependency.

Receiver operator characteristic (ROC) curves were performed to determine the optimum cut-off value and to evaluate the diagnostic accuracy.

Receiver operator characteristic curves of sensitivity vs. 1-specificity were plotted. This screening test is considered useless if the area under the curve (AUC) is less than 0.5. A screening test's performance is considered good if the AUC is between 0.7 and 0.8, excellent if the AUC is between 0.8 and 0.9, and outstanding if the AUC is >0.9.

All statistical analyses were performed using the Statistix™ software version and the criterion of significance was $P < 0.05$.

TABLE 1 | Clinical Characteristics of the studied population.

	Uncomplicated pregnancies	Preeclampsia	*Gestational hypertension*	IUGR without preeclampsia
n	810	40	33	20
Maternal age (years)	24.94 ± 6.41	26.6 ± 6.9	29.87 ± 9.07	25.95 ± 7.59
Gestational age (weeks)[1]	38.72 ± 1.33	36.75 ± 2.88	38.1 ± 2.2	35.8 ± 3.75
Birth weight (g)	3320.9 ± 455.6	2871.6 ± 968.2	2978.0 ± 814.3	2091.8 ± 569.23***
Body mass index (BMI), kg/m2	25.1 ± 5.9	28.5 ± 7.9	29.21 ± 18.9	23.03 ± 5.54
Sistolic blood pressure (mmHg)	110.0 ± 4.1	158.6 ± 6.7***	157.0 ± 4.3***	116.2 ± 3.5
Diastolic blood pressure (mmHg)	63.1 ± 2.5	107.0 ± 3.8***	101.1 ± 9.4***	67.3 ± 6.2
Proteinuria[2]	*Negative*	+	*Negative*	*Negative*

Values are mean ± SD.
****P < 0.001 compared to uncomplicated pregnancies.*
[1]Weeks from last menstrual period.
[2]Proteins in urine were determined by Test Urine Labstix Strip.

TABLE 2 | Characteristics of studied preeclamptic population accordingly the different presentations of preeclampsia.

Preeclampsia	*n*	Gestational age (weeks)	Birth weight (g)
Group A Gestational age <34 wks	5	30.19 ± 2.48	1440.6 ± 737.4
Group B Gestational age between 34 and 36.9 wks	13	35.44 ± 0.78	2331.5 ± 501.0
Group C Gestational age >37 wks with fetal growth restriction	5	37.8 ± 0.75	2405.8 ± 94.7
Group D Gestational age >37 wks without fetal growth restriction	17	38.48 ± 0.96	3276.7 ± 482.7

Values are mean ± SD.

RESULTS

Of the 1,293 pregnant women who received full antenatal care at the hospital enrolled in this study, only 1,256 were single pregnancies. Among these women, 810 were normotensive pregnant women without any other type of pathology, 40 presented preeclampsia and 33 gestational hypertension, and 20 IUGR without preeclampsia.

Tables 1, **2** show the clinical characteristics of the patients included in this study.

First, we evaluated the serum uric acid levels during gestation. As previously described, serum uric acid levels in preeclamptic women significantly increased, compared to uncomplicated pregnancies, until the end of pregnancy. In women with gestational hypertension uric acid levels tended to rise toward the 35th week of gestation, however, this increase is not statistically significant ($P = 0.068$). In contrast, the uric acid levels in women who presented IUGR without preeclampsia were similar to those observed in non-pathological pregnancies (**Figure 1**).

No association was found between UAr and BMI ($p = 0.404$) and UAr and parity ($p = 0.409$). Although the interaction between UAr and maternal age was statistically significant ($p = 0.011$), Phi coefficient (0.092), Cramer's V (0.092), and the contingency coefficient (0.091) showed no association between the UAr and maternal age.

Interestingly, the serum urea and creatinine dosages were normal throughout pregnancy in all the groups (**Table 3**). Therefore, renal failure may not be responsible for the increase in uric acid in preeclamptic pregnant women.

We also analyzed the uricemia ratio and as we expected, it was >1.5 in women with preeclampsia. We also observed that UAr was also >1.5 in women who had gestational hypertension (**Table 4**), suggesting a link between uric acid and high blood pressure.

Then, we deconstructed the group of preeclamptic pregnant women accordingly to the onset of the clinical manifestations. We found that Groups A and C, which are the most severe

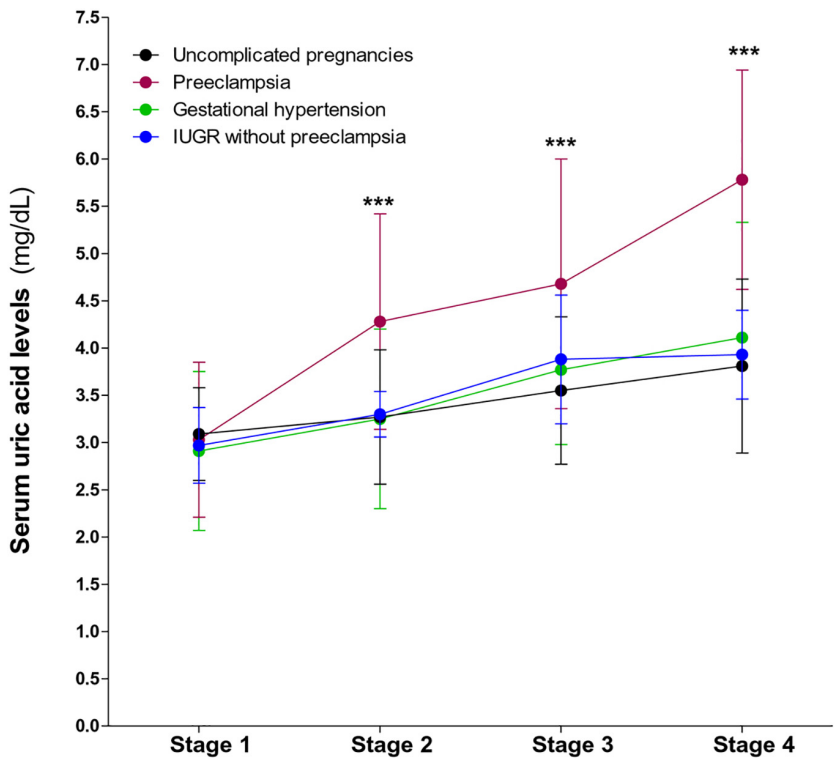


FIGURE 1 | Behavior of serum uric acid levels throughout gestation in preeclampsia, gestational hypertension, IUGR without preeclampsia, and in non-complicated pregnancies. A significant increase in uric acid levels is observed in preeclampsia. Values are mean ± SD. ***$P < 0.001$ preeclampsia vs. uncomplicated pregnancies.

TABLE 3 | Serum urea and creatinine levels in the studied population.

	n	Creatinine levels (mg/dL)		Urea levels (mg/dl)	
		Before 20 wks of gestation	After 20 wks of gestation	Before 20 wks of gestation	After 20 wks of gestation
Uncomplicated pregnancies	810	0.52 ± 0.09	0.50 ± 0.09	0.18 ± 0.06	0.17 ± 0.07
Preeclampsia	40	0.54 ± 0.12	0.56 ± 0.13	0.17 ± 0.06	0.20 ± 0.07
Gestational hypertension	33	0.55 ± 0.12	0.54 ± 0.19	0.16 ± 0.07	0.15 ± 0.05
IUGR without preeclampsia	20	0.57 ± 0.11	0.63 ± 0.23	0.18 ± 0.05	0.20 ± 0.06

Values are mean ± SD. In all cases, serum urea and creatinine levels did not change throughout gestation ($P > 0.05$).

presentations of preeclampsia, showed drastic increases in serum uric acid levels between 20 and 30 weeks of gestation with a UAr greater than 1.5. On the other hand, Groups B and D showed a significant increase in uric acid levels after 31 weeks of gestation resulting in UAr higher than 1.5 (**Table 5**).

Then, we compared uric acid levels in IUGR pregnancies with and without preeclampsia. Interestingly, uric acid levels only increased when the fetal growth restriction was associated with preeclampsia showing an UAr greater than 1.5 (**Table 6**).

TABLE 4 | Uric acid ratio (UAr) in uncomplicated pregnancies, preeclampsia, gestational hypertension, and IUGR without preeclampsia.

	Uric acid ratio (UAr)		
	Stage 2/1	Stage 3/1	Stage 4/1
Uncomplicated pregnancies	1.12 ± 0.02	1.22 ± 0.02	1.31 ± 0.03
Preeclampsia	1.63 ± 0.18	1.79 ± 0.21	2.17 ± 0.22
Gestational hypertension	1.32 ± 0.18	1.49 ± 0.17	1.67 ± 0.22
IUGR without preeclampsia	1.14 ± 0.07	1.36 ± 0.02	1.37 ± 0.11

Uric acid ratio was >1.5 in women with preeclampsia since the early stages of gestation. In women with gestational hypertension, UAr was >1.5 at the end of gestation. In non-complicated pregnancies and IUGR without preeclampsia, UAr was <1.5 during all gestation. Values are mean ± SEM.

TABLE 5 | Uric acid ratio (UAr) in women who developed different presentations of preeclampsia.

	Uric acid ratio (UAr)		
	Stage 2/1	Stage 3/1	Stage 4/1
Uncomplicated pregnancies	1.12 ± 0.02	1.22 ± 0.02	1.31 ± 0.03
Preeclampsia Group A	1.85 ± 0.47	2.33 ± 0.47	–
Preeclampsia Group B	1.30 ± 0.26	1.91 ± 0.38	1.86 ± 0.26
Preeclampsia Group C	1.83 ± 0.56	1.96 ± 0.58	2.38 ± 0.57
Preeclampsia Group D	1.51 ± 0.28	1.70 ± 0.28	2.07 ± 0.34

Groups A and C showed UAr >1.5 during all gestation. On the other hand, Groups B and D showed UAr >1.5 after 30 weeks of gestation. Values are mean ± SEM.

TABLE 6 | Uric acid ratio (UAr) in IUGR pregnancies with or without preeclampsia.

	Uric acid ratio (UAr)		
	Stage 2/1	Stage 3/1	Stage 4/1
IUGR without preeclampsia	1.14 ± 0.07	1.36 ± 0.02	1.37 ± 0.11
IUGR with preeclampsia	1.84 ± 0.27	1.97 ± 0.33	2.44 ± 0.41

In IUGR pregnancies without preeclampsia, UAr was <1.5 along gestation. In contrast, in IUGR pregnancies with preeclampsia, UAr was higher than 1.5 since the early stages of gestation. Values are mean ± SEM.

Finally, we studied the diagnostic performance of serum uric acid as a biomarker of preeclampsia. ROC curves were constructed with uricemia ratios (uricemia after the 20th week of gestation/uricemia before the 20th week of gestation) in patients with preeclampsia and normotensive pregnant women without any pathology (**Figure 2A**). Sensitivity and specificity were calculated for each cut-off point. The cut-off point with maximum sensitivity and specificity was equal to 1.50. With these data, the area under the curve (AUC), the Positive Predictive Values (PPV) Negative Predictive Values (NPV), and the probability indices (LR) were calculated. We also contrasted ROC curves for the Group A of preeclampsia in which the clinical manifestations occur before 34 weeks of gestation (**Figure 2B**).

Our results showed the AUC was 0.918 [95% confidence interval (CI): 0.858–0.979) for the preeclampsia group and 0.955 (95% CI: 0.908–1.000) for Group A of preeclampsia, showing that the uricemia ratio can be considered as a screening test. However, a UAr at a cut-off point ≥1.5 had a very low positive predictive value. Interestingly, it had a high negative predictive value of 99.5% for preeclampsia and it reaches 100% for Group A of preeclampsia.

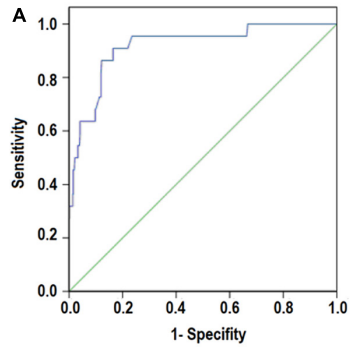
DISCUSSION

Hypertensive disorders of pregnancy are a relevant medical problem that affects a high number of pregnant women, being associated with the development of both maternal and fetal complications. Within these disorders, preeclampsia constitutes the most severe of the hypertensive complications of pregnancy.

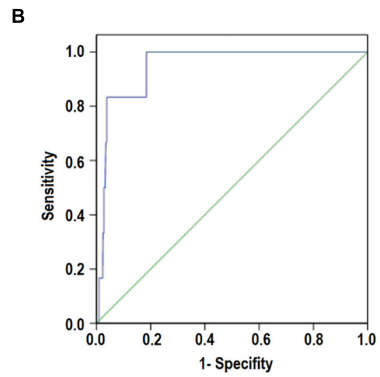
Currently, many molecules have been proposed as biomarkers for preeclampsia, although their usefulness and versatility are controversial. Among them, angiogenic markers such as s-Eng or s-Flt-1/PlGF ratio have been demonstrated to be the most promising (Verlohren et al., 2012; Brownfoot et al., 2017; Dröge et al., 2021). Unfortunately, the clinical use of these biomarkers requires expensive and sophisticated technologies. The implementation of these tests as a routine medical practice in all pregnant women seems not to be feasible, particularly in developing countries of Latin America, Asia, or Africa, with a low-income socioeconomic environment.

On the other hand, the role of uric acid in preeclampsia has generated growing interest.

Although the link between increased uric acid levels and preeclampsia is well-known, the clinical value of this association is still up for discussion. The classical interpretation of the increase in serum uric acid levels proposes that vasoconstriction



AUC (95% CI)	Sensitivity %	Specificity %	+LR	−LR	PPV %	NPV %
0.918 (0.858-0.979)	90.9	83.6	5.54	0.11	19.2	99.5



AUC (95% CI)	Sensitivity %	Specificity %	+LR	−LR	PPV %	NPV %
0.959 (0.908-1.000)	100	81.6	5.43	0.001	4.7	100

FIGURE 2 | Receiver operating curves (ROC) curves to determine the diagnostic utility of serum uric acid ratio in **(A)** Preeclampsia **(B)** Preeclampsia Group A (who develop preeclampsia before 34 weeks of gestation). AUCs for both curves were >0.9 showing that UAr may be a good screening tool. However, it has very low positive predictive values (19.2 and 4.7, respectively). In contrast, the negative predictive values were 99.5% for preeclampsia and 100% for Group A of preeclampsia. Area under the curve (AUC), confidence interval (CI), Positive Predictive Values (PPV) Negative Predictive Values (NPV), probability indices (LR).

induced by hypertension causes a decrease in its renal clearance (Many et al., 1996).

With growing knowledge of the effects of uric acid on the endothelium, oxidative stress, and inflammation, features that are assumed to have a role in the pathogenesis of pre-eclampsia, the interest in the significance of uric acid as a prognostic marker of preeclampsia has been renewed (Mulla et al., 2011; Matias et al., 2015).

There are studies in the literature that support the use of uricemia as a predictor of this pathology (Many et al., 1996; Roberts et al., 2005; Bellos et al., 2020), but there are also others that criticize it arguing that its positive diagnostic value is not optimal. Even more, it was also proposed that increased uric acid levels have been linked to poor maternal and fetal outcomes in several studies (Ugwuanyi et al., 2021; Zhao et al., 2021). Nevertheless, other studies stated that a high uric acid level is an unreliable predictor of maternal and fetal outcomes (Thangaratinam et al., 2006; Chen et al., 2016; Pecoraro and Trenti, 2020).

In this work, we observed that during uncomplicated pregnancies the values of uric acid levels decrease between 25 and 35% at the beginning of gestation, showing lower values than in non-pregnant women, and then it increases slightly and reach toward the end of gestation similar values to those of non-pregnant women. This behavior could be attributed to estrogens' uricosuric action (Nicholls et al., 1973). Since uricemia levels rise toward the end of pregnancy, this uricosuric impact must be addressed by some regulatory mechanism or by an overproduction of this metabolite. This rise could be also explained by the inflammatory reaction that occurs when labor begins (Romero et al., 2018).

Because of the biological variability of uricemia, we have previously proposed the calculation of the UAr (serum uric acid levels after 20 weeks of gestation/uric acid levels before 20 weeks of gestation) as an analysis tool. We proposed that a ratio greater than 1.5 would be related to the onset of preeclampsia (Corominas et al., 2014). Here, we conducted a prospective study to evaluate the diagnostic value of this uricemia ratio as a predictive marker of preeclampsia. Although one of the limitations of our study was the low number of preeclamptic women included in the different subgroups, it is in concordance with the incidence of preeclampsia in our country (Corominas et al., 2014; Guevel, 2018).

We found that in all presentations of preeclampsia, serum uric acid levels increased. However, the time of the rise of uric acid levels depended on the severity of the disease. Thus, in women who develop preeclampsia before 34 weeks (Group A) or in those who develop preeclampsia after 37 weeks associated

with IUGR (Group C), uric acid levels significantly raised after week 20 weeks of gestation. On the other hand, in women who develop preeclampsia after 34 weeks without IUGR, uric acid levels increased later in gestation (Groups B and C).

These findings proposed that the timing of the increase of serum uric acid and the magnitude of the increase (represented by the UAr) may be associated with the severity of the condition.

On the other hand, although in gestational hypertension uric acid levels showed a slight increase near to term, this rise was not statistically significant, possibly due to the small size of the sample. However, in these women, UAr was greater than 1.5 toward the end of gestation.

One of the major weaknesses of many of the potential biomarkers for preeclampsia is the difficulty in discerning between IUGR with preeclampsia and without preeclampsia (Litwińska et al., 2017; Huppertz, 2020). In this regard, our findings revealed that when the fetal growth restriction is not associated with preeclampsia, uric acid levels do not increase throughout pregnancy. Therefore, these results may support the hypothesis that the rise of uric acid levels is linked to maternal endothelial dysfunction and exacerbated systemic inflammatory response in preeclampsia (Huppertz, 2020; Redman et al., 2021). In this regard, we propose that monitoring uric acid levels during pregnancy, in combination with biochemical and ultrasonographic markers, could enable a better diagnosis of these disorders.

Based on ROC curves, we also demonstrated that the uricemia ratio (serum uric levels after 20 weeks of gestation/serum uric levels before 20 weeks of gestation) has diagnostic value. Regarding this, we found that a UAr less than 1.5 is a helpful parameter with a strong exclusion value and high sensitivity for those women who are not expected to develop preeclampsia. Even though it is uncommon to employ an analyte dosage as an exclusion tool, our results confirmed the clinical value of monitoring the uric acid levels during gestation as a "collaborator" in the identification and prediction of preeclampsia.

Although many studies have ruled out the value of uric acid as a predictor of preeclampsia in the first trimester, its evaluation in the second and third trimesters would still provide useful information to timely referring a woman at risk to a more complex center. The hunt for first trimester biomarkers does not solve the problem in our region, where about half of all women still go to their initial check-up after the first trimester (Corominas et al., 2014; Giachini et al., 2017).

In this sense, a marker that allows excluding those women who are not at risk of preeclampsia from those who potentially are would let us conduct a more exhaustive follow-up of these patients at risk and the opportune referral to a health center of more complexity. Thus, this would not only serve for the administration of effective prophylactic therapies to prevent the progression of the disease and improve perinatal obstetric outcomes but also for the minimization of the offspring's long-term complications.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics Committee of the Hospital Nacional Profesor Alejandro Posadas, Buenos Aires, Argentina. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AC and YM carried out the experimental work and analysis of data. SB, RC, MF, and NM carried out the data analysis and discussion and critically reviewed the manuscript. AD designed the study and wrote the manuscript. All authors contributed to the final version of the manuscript.

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Melatonin Hormone Acts on Cells of Maternal Blood and Placenta From Diabetic Mothers

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Changes in glucose metabolism of diabetic mothers affect immunological components, proinflammatory factors, and placental hypervascularization that can induce cell death. The hormone melatonin has been identified as a potential modulating agent. The aim of this study was to analyze the oxidative process and the apoptosis in maternal blood and placental cells modulated by melatonin from diabetic mothers. The groups were 40 pregnant women divided into non-diabetic (ND) and type 2 diabetes mellitus (T2DM) groups. Blood and placental cells were obtained by density gradient and maintained in culture treated or not with melatonin (100 ng/mL) for 24 h (37°C, 5% CO_2). Oxidative stress was evaluated by superoxide release and CuZn superoxide dismutase (SOD). Apoptosis was assessed by flow cytometry. Maternal hyperglycemia increased superoxide release and apoptosis in MN cells from maternal blood and reduced SOD level and SOD/O2- ratio. Melatonin reduced oxidative stress and apoptosis rates in MN cells in the blood of diabetic mothers. There was a reduction in SOD and SOD/O2- ratio in the placental extravillous layer, and melatonin restored the concentrations of this enzyme. There was greater superoxide release, reduced SOD/O2- ratio, and apoptosis in MN cells placental villous layer. Melatonin increased apoptosis rates in the placental villous layer from hyperglycemic mothers. These data suggest that hyperglycemia altered the processes oxidative in blood and placenta from hyperglycemic mothers. These changes reflected in the mechanisms of induction of apoptosis, especially in the vascularized layers of the placenta, and were modulated by melatonin.

Keywords: apoptosis, mononuclear cells, diabetes, melatonin, placenta, blood

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INTRODUCTION

The placenta is a highly vascularized transitory organ from fetal–maternal tissues that play important functions in maintaining pregnancy and promoting fetal development (Moore and Persaud, 2008). This organ plays a critical role in immunoregulation (Hara et al., 2016) and sources cells from maternal and fetal origin. These cells present molecules that have an essential role in maternal–fetal tolerance (Guleria and Sayegh, 2007).

The placenta can alter cell and cytokine levels from maternal blood before passing them on to the fetus. The inflammatory environment influences this mechanism due to hyperglycemia

(Hara et al., 2016) that causes many major complications macro-and microvascular damage (American Diabetes Association [ADA], 2014).

Maternal hyperglycemia may trigger structural and physiological responses to assure maternal–fetal exchanges and fetal oxygen delivery and alter cellular oxidative metabolism at the maternal–fetal interface (Gauster et al., 2011) with an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense.

These changes cause modification of cellular proteins, lipids, and DNA, affecting cellular behavior and differentiation and inducing apoptosis and cellular damage (Espinosa-Diez et al., 2015). In addition, in the placenta villous layer of diabetic mothers, memory T cells and Fas expression are reduced, which may alter T cell apoptosis and regulate maternal–fetal immune tolerance (Queiroz et al., 2019).

The placenta, through maternal blood, establishes an interface for the exchange of nutrients and gases with the fetus, and the production of specific regulatory molecules with metabolic and endocrine activities, with the participation of several hormones (Benirschke, 2000). For example, the hormone melatonin has effects on pancreatic insulin secretion (Fu et al., 2013) and cellular oxidative metabolism (Fernandes et al., 2019), and antioxidant action (Arendt and Skene, 2005; Morceli et al., 2013).

There is evidence of the importance of melatonin as a potent immunomodulatory agent that improves the functional activity of cells (França et al., 2008; Honorio-França et al., 2009, Honorio-França et al., 2013). In addition, melatonin can bind to phagocytes and trigger various oxidative processes in the body (Fernandes et al., 2019).

It is known that melatonin stimulates the release of active oxygen metabolites by immune cells (Honorio-França et al., 2013). The mechanisms by which melatonin influences immune functions involve the participation of other hormones, cytokines, and specific receptors (Pandi-Perumal et al., 2008). Studies with macrophages in experimental models of diabetes and diabetic patients report that melatonin exerts antioxidant action. In animal cells and non-diabetic individuals, it has a pro-oxidative effect (França et al., 2008; Honorio-França et al., 2009; Morceli et al., 2013). However, the effects of melatonin on the oxidative process on placental cells and their action on the maternal–fetal interface have not yet been elucidated. Melatonin may act in the cellular oxidative process by favoring the development of the fetus and the vascularization of the uteroplacental in pregnancies of diabetic mothers. Thus, this study proposed to analyze the cellular oxidative process and the induction of apoptosis in maternal blood and placental cells modulated by melatonin from diabetic mothers.

MATERIALS AND METHODS

A cross-sectional study was performed with blood and placenta from diabetic mothers. The mothers were attended the Diabetes and Pregnancy Facility, School of Medicine Obstetrics Course, UNESP, Botucatu, SP. The local Research Ethics Committee approved this study, and all the women gave informed written consent.

Subjects

For analysis of blood and placenta, the pregnant women (18–45 years old) were separated by maternal glycemic status. Pregnant women with diabetes mellitus type 2 (T2DM) were referred to the service with a confirmed diagnosis. The non-diabetic pregnant women were evaluated by a 75-g oral glucose tolerance test (OGTT-75 g-American Diabetes Association [ADA], 2014) and glucose profile (GP; Rudge et al., 2000; Calderon et al., 2007) between the 24th and 28th weeks of pregnancy. Thus, according to the test results, 40 pregnant women were classified into the following groups: the non-diabetic (ND) group (normal 75 g-OGTT and normal GP; n = 20) and the type 2 diabetes mellitus (T2DM) group (abnormal GTT-75 g, prior to pregnancy n = 20) (American Diabetes Association [ADA], 2014).

Independent of diagnosis, the pregnant women continued attending the service. Patients with T2DM were evaluated every 2 weeks until the 32nd week for GP with fasting, pre-and post-prandial glycemic levels for 24 h. In addition, they were treated with physical exercise, a specific diet, and insulin therapy from the beginning of the pregnancy until delivery (Calderon et al., 2007) for glycemic control (Rudge et al., 2000). A glycaemic mean of 120 mg/dl or less was defined as adequate glycaemic control, and a glycaemic mean higher than 120 mg/dL was defined as inadequate glycaemic control. The ND pregnant women did not receive any type of therapy for glycemic control.

These patients were individualized by gestational age at delivery, altered blood pressure, and body mass indexes. In addition, women with gestational age until 20 weeks who received prenatal and delivery care at the Service and signed a Consent Form and delivered in the morning were considered inclusion criteria. The exclusion criteria were women with multiple pregnancies, T1DM, GDM, fetal malformations, and deliveries before the 34th week of gestation.

Blood Sampling and Separation of Blood Cells

Before labor, approximately 8 mL of blood sample was collected into heparinized (25 U/ml) tubes in the morning (8:00 at 10:00 h) at the 36th week of pregnancy. The plasma was retired and maintained at −80°C for the determination of glucose, melatonin, and SOD. The cells were obtained by fractionated with Ficoll-Paque density gradient (density 1.077 g/L; centrifugation 160 × g; 40 min; Pharmacia, Upsala, Sweden) and resuspended independently in serum-free medium 199 at a final concentration of 2 × 106 cells mL^{-1} and used immediately for assays of superoxide release and apoptosis.

Placenta Sampling and Separation of Cells

Placenta was obtained at delivery and washed with saline solution. A sample was collected along the placenta to remove the villous region with a margin of approximately 2 cm

from the insertion of the umbilical cord, midway between the maternal and fetal sides. The large vessels were removed, leaving only the villous tissue (22,23). The basal plate was carefully dissected from the villous tissue and amniochorium membrane. The isolation of extravillous tissue was adapted from the procedure described for isolating amniochoric cytotrophoblast and removing the extravillous region from the placental border (Calderon et al., 2007; Stenqvist et al., 2008; Vincent et al., 2015). The fragments were stored in liquid nitrogen for later obtaining of cells. The samples (100 mg of tissue/ml) were macerated in PBS with Tween 20 supplemented with protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride; 0.1 mM benzethonium chloride, 10 mM EDTA, 20 UI aprotinin, and 0.5% BSA) using a homogenizer Power Gen 125 (Fisher Scientific@). The homogenate was filtered and reserved for MLT and SOD determination, and the sediment (cells) was fractioned by Ficoll-Paque gradient (density 1.077 g/L; centrifugation 160 × *g*; 40 min; Pharmacia, Upsala, Sweden). After centrifugation, the cells were collected using a siliconized Pasteur pipet and transferred to tubes. The cells were washed twice with Medium 199 (Sigma Chemical, St. Louis, MO, United States) for superoxide release and apoptosis assays.

Glucose Determination

Glucose levels were quantified by the glucose oxidase method (Glucose – analyzer II Beckman, Fullerton, CA, United States). HbA1c was determined by high-performance liquid chromatography (D10^{TM} hemoglobin testing system, BIO-RAD Laboratories, Hercules, CA, United States).

Determination of Melatonin

The hormone melatonin (plasma and placenta homogenate) was quantified by the Melatonin ELISA kit (IBLHamburg, German). The kit has the following characteristics: the lower detection limit was 1.6 pg/ml, and intra-assay and inter-assay coefficients of variation (%) were 3.0–11.4 and 6.4–19.3, respectively. Melatonin extraction was performed by the affinity chromatography method using standardized columns. Columns were placed in glass tubes and centrifuged twice with 1 ml methanol (1 min – 200 × *g*). Then the columns were washed two times with double distilled water (1 min – 200 × *g*). After preparation of columns, 0.5 ml of standards, controls, and samples were applied and centrifuged for 1 min at 200 × *g*. After applying the samples and standards, the columns were washed again with 1.0 ml of 10% methanol for 1 min at 500 × *g*. Next, the extraction of the eluate containing the hormone melatonin was carried out by adding 1.0 ml of methanol at 200 × *g*. After obtaining the eluate, the methanol was evaporated using an evaporator centrifuge (speed-vac). The material was reconstituted with 0.15 ml of double distilled water under stirring for 1 min and immediately analyzed. 50 ml of each standard, control, and colostrum and milk samples were placed in an ELISA plate with 50 ml of melatonin-biotin in each well with 50 ml of antiserum. The plate was incubated at 4°C for 20 h. After this period, the supernatant was discarded, the plate was washed 3 times with wash buffer, and 150 ml of the conjugated enzyme was added. After 120 min of incubation at room temperature, the plate was washed 3 times, 200 ml of the substrate p-nitrophenyl phosphatase (PNPP), and incubated for another 40 min under agitation. After this period, the reaction was blocked with 50 ml of "PNPP stop" solution, and the reading was done in a spectrophotometer for a 405 nm filter. Results were calculated using the standard curve (R^2 = 0,984) and expressed in pg/mL (Honorio-França et al., 2013).

Cells Treatment

Blood and placental MN cells treated or not with 50 μL of melatonin (MLT −100 ng/mL final concentration- Honorio-França et al., 2013) were incubated for 24 h (37°C; 5% CO_2). After the cells were resuspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS-Sigma, St. Louis, MO, United States), the cells were tested for superoxide release and apoptosis. The culture supernatant was reserved for quantitation of the superoxide enzyme (SOD). For each assay performed, a phagocyte control (2 × 10^6 cells/ml) was incubated for a similar time, depending on the type of essay in medium 199 or PBS, in the absence of melatonin.

Superoxide Anion Determination

Superoxide release was evaluated by method reduction of cytochrome C (Sigma, St Louis, MO, United States; Honorio-França et al., 1997). Blood and placental MN cells treated or not with MLT were incubated for 24 h. After this time, the cells were centrifuged (160 × *g*, 10 min) and resuspended in PBS containing 2.6 mM $CaCl_2$, 2 mM $MgCl_2$, and cytochrome C. The cells (100 μL) were then incubated on culture plates at 37°C for 1 h. A control of untreated cells was used to evaluate spontaneous release. The reactions were measured by absorbance at 550 nm, and the results are expressed as nmol. O_2^-. The experiments were performed in duplicate.

TABLE 1 | Clinical data on the pregnant women non-diabetic (ND) and T2DM (type 2 diabetes mellitus).

Parameters	ND	T2DM
Age (years)	27,1 ± 3.8	29.5 ± 5.3
Gestacional Age (weeks)	38,0 ± 1.2	37.8 ± 0.8
Glucose level (mmol/L)	4,2 ± 0.7	5.9 ± 0.8*
HbA1c (%)	5,2 ± 0.4	6.4 ± 0.8*
BMI-1	27,2 ± 4.2	30.3 ± 6.0
BMI-2	31,8 ± 7.3	34.4 ± 8.9
Hypertension	10%	40%#
Smoking	10%	5%#
Physical exercise	25%	70%#
Placental weight (g)	612.5 ± 99.4	716.6 ± 112.6
Placental Index	0.162 ± 0.035	0.179 ± 0.031*

HbA1c - Glycated hemoglobin; BMI-1 and BMI-2 (body mass index in the first and third trimesters of pregnancy, respectively).
Data correspond to the median of 40 mothers.
The placental index is the ratio of placental weight to fetal weight.
**P < 0.05 statistical difference (Student's t-test).*
#P < 0.05 statistical difference (Chi-square test).

CuZn-Superoxide Dismutase Determination (CuZn-Superoxide Dismutase–E.C.1.15.1.1)

Analysis of the CuZn-SOD enzyme was determined in plasma, placenta homogenate, and cell culture supernatants treated or not with melatonin from maternal blood and placenta using the nitroblue tetrazolium (NBT) reduction method (Sigma, St Louis, MO, United States; Novelli et al., 1993). The individual samples (0.5 mL) and standard (hydro-alcoholic solution) were placed in glass tubes. Next, 0.5 mL of chloroform-ethanol solution (1:1 ratio), 0.5 mL of reactive mixture (NBT and EDTA) and 2.0 mL of buffer carbonate/hydroxylamine ($pH = 10.2$) were added. The tubes remained still at room temperature for 15 min and were subsequently read at 560 nm. The results were expressed in international units (IU) of CuZn-SOD and were calculated by the following equation:

$$\text{CuZn-SOD} = (\text{Ab standard} - \text{Ab sample}/\text{Ab standard}) \times 100 = \%\ \text{reduction of NBT}/\text{CuZn-SOD}.$$

Apoptosis Assay

To determine the apoptosis rates, and APO-DIRECT^{TM} kit (BD Biosciences - United States) was used. As per the manufacturer's instructions, apoptosis tests were performed. The flow cytometry (FACSCalibur system; BD, San Jose, United States) was used to analyze the results, and the data were evaluated by Cell Quest software.

Statistical Analysis

The Student's *t*-test was used to evaluate the age, gestational age, BMI, placental weight and index, glucose, glycated hemoglobin, melatonin, and superoxide dismutase. The chi-square test analyzed qualitative variables (hypertension, smoking, and physical exercise). Analysis of variance (one-way ANOVA) and Tukey's tests were used to evaluate the superoxide release, superoxide dismutase (in culture), and apoptosis index of MN cells from blood and placenta treated or not with melatonin. Statistical significance was considered when $p < 0.05$.

RESULTS

Clinical data of the mothers (ND and T2DM) are shown in **Table 1**. Pregnant women showed similarities in gestational age at birth, maternal age, and weight before pregnancy. Glycated hemoglobin levels were increased in diabetic mothers (**Table 1**).

The melatonin and superoxide dismutase (SOD) levels were evaluated in both groups' maternal blood and placenta (**Figure 1**). The melatonin and SOD levels were higher in the maternal blood from diabetic mothers (**Figure 1A**). In placenta villi, both melatonin and SOD were lower in the diabetic group than in the ND group (**Figure 1B**). In contrast, in the placental extravillous layer, melatonin and SOD were similar among the groups (**Figure 1C**).

In culture supernatants from maternal blood MN cells, the SOD levels were lower in the diabetic group. Similar concentrations of the enzyme were observed in cells treated with melatonin in both studied groups. Similar SOD levels were later observed in MN cells in the placenta villi, irrespective of melatonin treatment. A reduction in SOD levels was observed in the MN cell culture supernatants from the placenta extravillous layer of diabetic mothers compared to ND mothers. Similar enzyme values were found in the culture supernatants when these cells were incubated with melatonin, regardless of the glycemic level of the study subjects (**Table 2**).

Superoxide release was higher in MN cells from maternal blood from diabetic mothers than in MN cells from ND mothers. In the non-diabetic group, blood MN cells stimulated with melatonin presented higher superoxide release than untreated cells ($p < 0.05$). However, melatonin did not increase superoxide release in blood MN cells in the hyperglycemic group (**Figure 2A**).

Irrespective of placental section, superoxide release was higher in MN cells from the placental villous layer of diabetic mothers. Melatonin increased superoxide release in cells from the placental villous layer (**Figure 2B**). However, superoxide release was lower in MN cells from placenta extravillous from the T2DM group (**Figure 2C**). The highest superoxide levels were observed in phagocytes of hormone-treated non-diabetic mothers (**Figure 2C**).

The placenta villous/extravillous superoxide release ratio is shown in **Figure 3A**. It was observed that there was an increase in the anion release ratio between the MN cells of the different placental layers in the diabetic group compared to the ND group. In contrast, melatonin treatment reduced the placenta villous/extravillous superoxide ratio.

Figure 3B shows the placenta villous/extravillous SOD ratios. There was a reduction of the enzyme in the placenta villous/extravillous ratio in the diabetic group compared to the ND group. However, there was a significant increase villous/extravillous SOD ratio when treated with melatonin.

The SOD/O_2^- ratio in the maternal blood and the placental villous and extravillous layers in both groups are shown in **Table 3**. Blood MN cells and placenta villous were lower in T2DM. Melatonin treatment increased the SOD/O_2^- ratio in the T2DM group with values similar to those found in normoglycemic mothers. There was no difference between the groups studied in the SOD/O_2^- ratio in the placental extravillous layer (**Table 3**).

Higher apoptosis rates were found in maternal blood cells from the diabetic group relative to the non-diabetic group. Melatonin treatment reduced the apoptosis rates in this group relative to the ND group. In the placenta villous, the apoptosis rates were lower in the T2DM group irrespective of melatonin stimulation. In both groups, there were no significant differences between the apoptosis indices of MN cells from placenta extravilli (**Table 4**).

DISCUSSION

Hyperglycemia maternal promotes the production of reactive oxygen species (ROS), resulting in oxidative stress, which

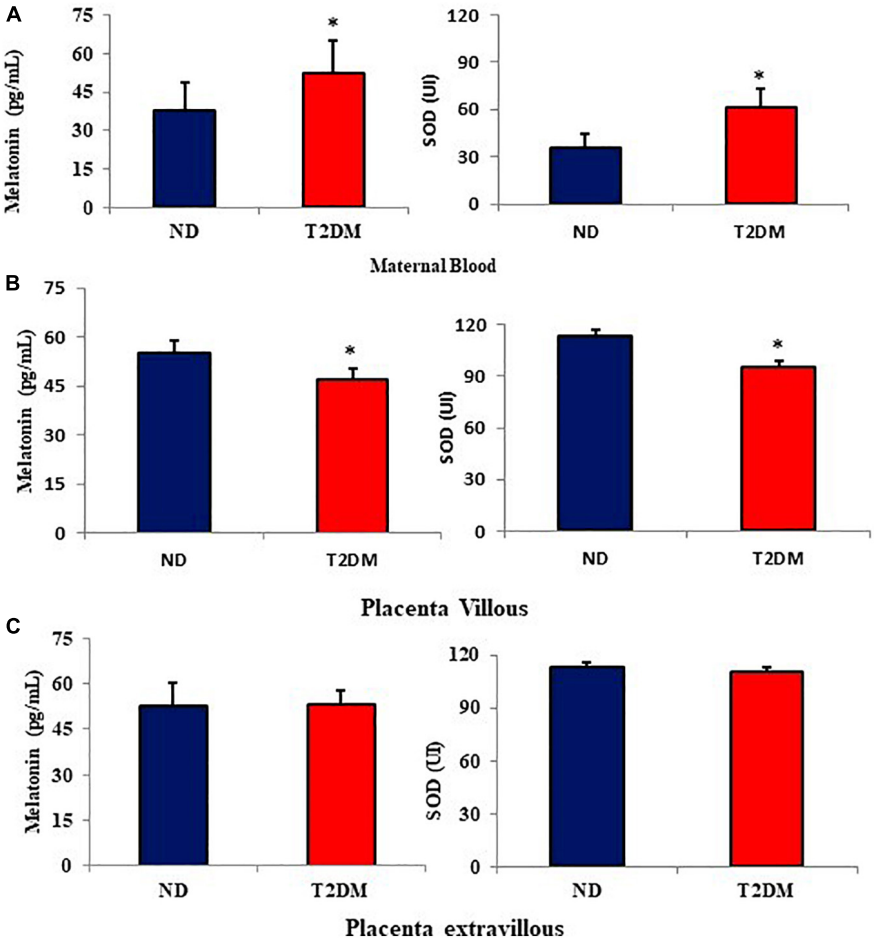

FIGURE 1 | Mean (±SD; *N* = 10) melatonin levels (pg/mL) and superoxide dismutase (SOD- UI) in the maternal blood **(A)**, placenta villous **(B)**, and placenta extravillous **(C)** from diabetic mothers. *$P < 0.05$ (Student's *t*-test) indicates the difference between normoglycemic and hyperglycemic groups.

can contribute to the proinflammatory environment typical of diabetes (Cvitic et al., 2014) and placental hypervascularization, with alterations in vasculogenesis and VEGF-R1 and R2 receptors (Pietro et al., 2010). In this work, hyperglycemia was able to alter the balance between superoxide anion production and the enzyme superoxide dismutase (SOD) in maternal blood and the placenta of diabetic mothers. These alterations were reflected in the mechanisms of induction of apoptosis and were modulated by the hormone melatonin.

TABLE 2 | Superoxide dismutase (SOD) levels in the culture supernatant of maternal blood cells, placenta villi, and placenta extravilli treated or not with melatonin from diabetic mothers.

SOD (UI)		ND	T2DM
Maternal blood	MLT (–)	14.6 ± 6.3	4.71 ± 1.2*
	MLT (+)	17.7 ± 7.9	18.2 ± 6.1#
Placenta villous	MLT (–)	18.3 ± 5.9	17.2 ± 3.4
	MLT (+)	15.8 ± 7.1	13. ± 6.8
Placenta extravillous	MLT (–)	20.2 ± 5.4	9.3 ± 5.7*†
	MLT (+)	21.9 ± 8.9	19.2 ± 8.3#

The results represent the mean and standard deviation of 10 samples.
MN, Mononuclear cells (MN); MLT, melatonin.
*$P < 0.05$ statistical difference (ANOVA test) * comparing the groups considering the same treatment and type of sample; # comparing untreated cells with cells treated with melatonin, considering the same group and sample; †comparing between villous and extravillous placental layers considering the same treatment and group.*

Melatonin assessed in the morning in diabetic mothers had higher levels in maternal blood and lower in placental villi. However, maternal blood and placenta melatonin concentrations of women are variable. Some authors report higher hormone concentration in the blood (Ejaz et al., 2021) and tissue (Lanoix et al., 2012), while others show lower levels (Bouchlariotou et al., 2014) similar to those valours found in blood and placenta in this study and colostrum (Morceli et al., 2013) from diabetic mothers.

The blood MN cells of diabetic mothers presented an increase in superoxide anion, lower release of the enzyme superoxide dismutase, and reduction in the SOD/O_2^- ratio, suggesting alterations in the balance between the prooxidant and antioxidant systems. The free radical generation by mononuclear cells, including the superoxide anion, is an important defense mechanism against infectious diseases (França et al., 2011;

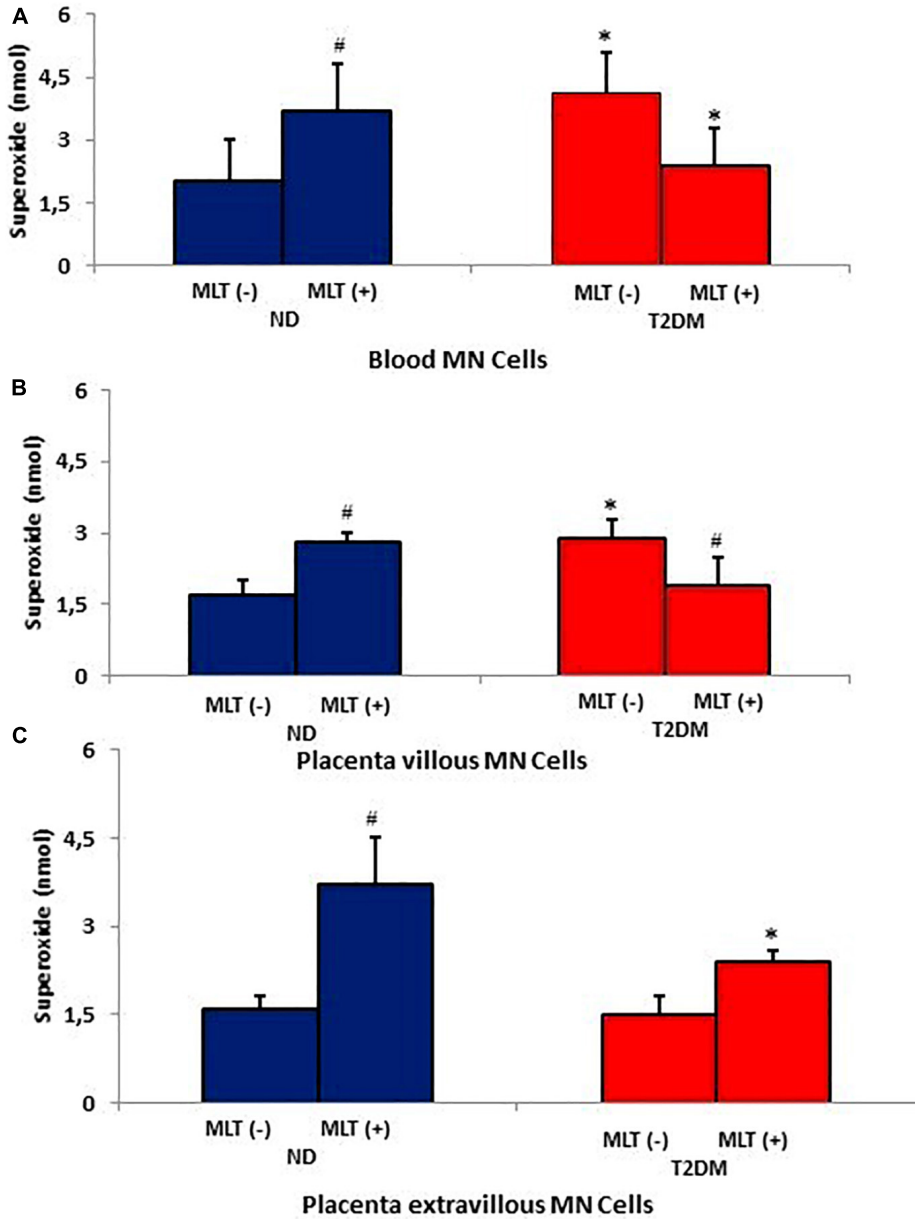

FIGURE 2 | Release of superoxide (O2-) by maternal blood phagocytes **(A)**, villous placenta **(B)**, and extravillous placenta **(C)** treated or not with melatonin from diabetic pregnant women. The results represent the mean and standard deviation of 10 MN cell samples from different mothers in each group. $P < 0.05$ statistical difference (ANOVA) * comparing the groups, considering the same treatment and type of sample; $^{\#}p < 0.05$, comparing cells treated with untreated melatonin, considering the same group and sample. Non-diabetic (ND); Type 2 Diabetes Mellitus (T2DM); melatonin (MLT); mononuclear cells (MN).

Fagundes et al., 2018), although the balance between the pro-and antioxidant mechanisms is also necessary since elevated levels of free radicals cause damage to cells that may result in the activation of cell death pathways (Benirschke, 2000; Maritim et al., 2003; Ferrari et al., 2011; Fernandes et al., 2019).

Various mechanisms have been proposed for the generation of reactive oxygen species. In diabetic patients, glucose oxidation may be the main source of free radicals (França et al., 2008; Ferrari et al., 2011). Hyperglycemia leads to lipid peroxidation by a superoxide-dependent pathway, resulting in the generation and release of free radicals (Rui et al., 2016). Additionally, the interaction of glucose with proteins that promote advanced glycosylation end products contributes to the excess free radical formation (Lappas et al., 2011).

Melatonin increased the release of superoxide in the non-diabetic group and reduced the release of this anion in MN cells of the diabetic group. This reduction affected the SOD/O_2^- ratio, resulting in values similar to those found in the control group.

The melatonin functions in humans are still partially understood. Melatonin can be associated with mononuclear

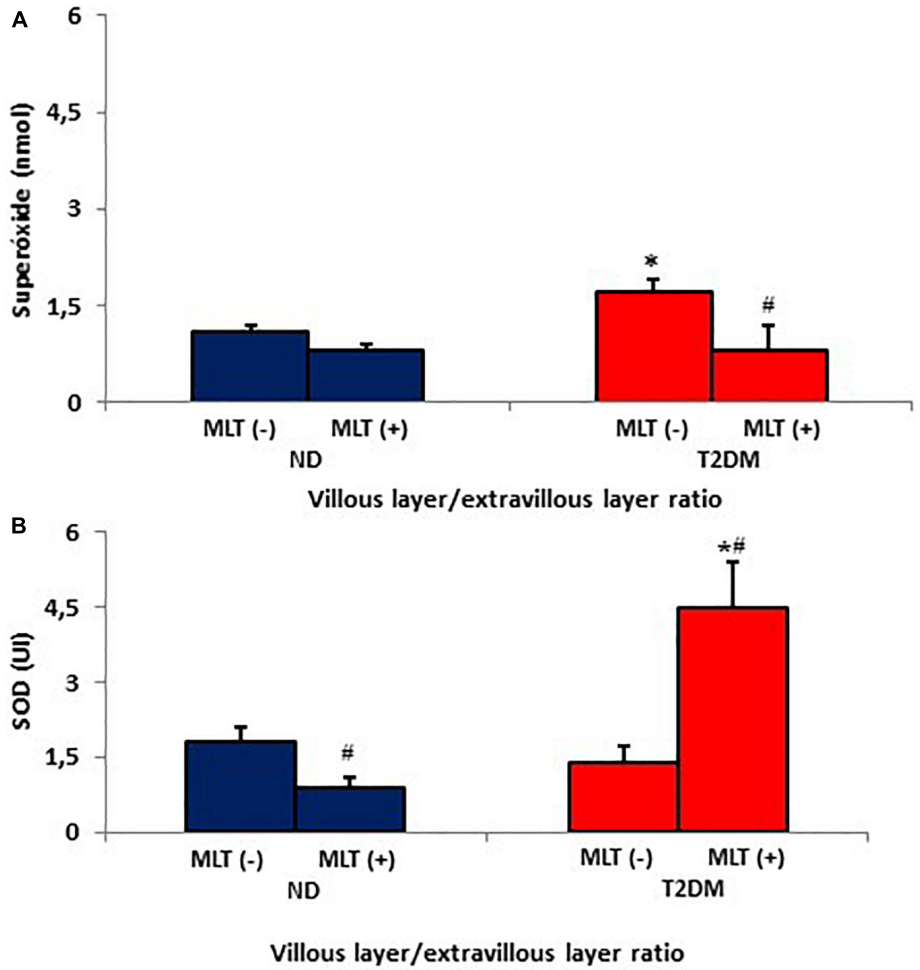

FIGURE 3 | Villous placenta/extravillous placenta ratios from the release of superoxide **(A)** and superoxide dismutase enzyme (SOD) **(B)** by MN cells of the non-diabetic (ND) and type II diabetes mellitus (T2DM) groups. The results represent the average and standard deviation of 10 samples. $P < 0.05$ statistical difference (ANOVA) * comparing groups considering the same treatment; $^{\#}p < 0.05$, comparing untreated cells with cells treated with melatonin, considering the same group.

cells, having even been related to the oxidative processes of the organism (França et al., 2008). Melatonin, depending on the dose administered, may have an antioxidative effect, including the scavenging of radicals (França et al., 2008) or pro-oxidative function (Hara et al., 2013; Honorio-França et al., 2013).

Melatonin increases superoxide production in MN phagocytes of non-diabetic mothers but not in the phagocytes of diabetic women. Maternal hyperglycemia alters the functional activity of these cells, and its effects are probably attributed to non-inflammatory processes, with lower superoxide release (Morceli et al., 2013). Inadequate stimulation of the activity of MN cells by melatonin, due to a failure in the prooxidant mechanisms, indicates an antioxidant effect related to diabetes (Reiter et al., 2008). Similar results were reported in diabetes models induced by the diabetogenic drug alloxan (Pawlak et al., 2005; Devi et al., 2008; França et al., 2008).

Alterations in the pro- and antioxidant mechanisms were also shown in the placenta being linked with its layers (extravillous and villous layers). The maternal layer showed a lower concentration of SOD enzyme. The presence of melatonin restored the levels of this enzyme with values similar to those of the control group. In addition, there was an increase in superoxide in the fetal layer and a reduction in the SOD/O_2 ratio. In the presence of melatonin, there was a reduction in superoxide. The SOD/O_2 ratio presented values similar to those of the control group, suggesting that in both layers of the placenta, melatonin could reduce oxidative stress, possibly caused by hyperglycemia.

Experimental studies in diabetic pregnancies have associated increased oxidative stress and reduced antioxidant capacity with abnormalities in the structure and function of the placenta (White et al., 2002). Thus, oxidative stress occurs in women with diabetic pregnancy and probably compromises antioxidant defense mechanisms and increases the generation of free radicals (Biri et al., 2009).

As a barrier between the mother and fetus, the placenta targets environmental changes (Radaelli et al., 2003). This organ can alter maternal blood cell and cytokine levels before transferring

them to the fetus, and the inflammatory environment influences this mechanism due to hyperglycemia (Hara et al., 2013). Further, there is an impact of maternal diabetes on fetal vascular growth and angiogenesis with placental hypervascularization (Cvitic et al., 2014). These changes in the fetoplacental vasculature in response to maternal diabetes may also imply potential differences in the fetus's vasculature. Maternal metabolic alterations resulting from hyperglycemia change the uterine environment and may lead to an abnormal fetal growth pattern (Catalano et al., 1999). The key to these alterations is maternal hyperglycemia, with consequent fetal hyperglycemia and hyperinsulinemia, inducing hypoxia, inflammation, and oxidative stress in the intrauterine environment. In this study, changes in oxidative stress found in maternal blood were reflected in the placenta and were probably directed to the fetus.

A relevant result is that the rate of superoxide release (placenta villous/extravillous) was higher in the placenta of T2DM, and melatonin was able to reduce this superoxide rate and increase the rate of SOD. Furthermore, it is known that melatonin can act in the removal of free radicals (Xia et al., 2019) and diabetes control (Sudnikovich et al., 2007) due to its beneficial action associated with the ability to eliminate free radicals and improve antioxidant activity (Sudnikovich et al., 2007; Pandi-Perumal et al., 2008; Xia et al., 2019). In this work, we suggest that melatonin modifies the placental tissue, generating an environment with antioxidant characteristics in the maternal–fetal interface and reinforcing the hypothesis that the placenta acts vicariously to protect the developing fetus.

TABLE 3 | Superoxide dismutase (SOD) and release of superoxide anion (O2-) rates (SOD/O_2^-) in the culture supernatant of MN cells of blood and placenta treated or not by melatonin of diabetic mothers.

Parameters		ND	T2DM
Maternal blood	MLT (–)	10.9 ± 4.7	2.7 ± 1.3*#
	MLT (+)	8.8 ± 3.7	11.0 ± 5.7
Placenta villous	MLT (–)	12.4 ± 4.3	4.1 ± 1.7*
	MLT (+)	14.0 ± 6.9	16.5 ± 5.7#
Placenta extravillous	MLT (–)	11.5 ± 3.5	11.6 ± 2.4†
	MLT (+)	9.6 ± 4.9†	7.5 ± 3.5#†

The results represent the mean and standard deviation of 10 samples.
MN, Mononuclear cells; MLT, melatonin (MLT).
*P < 0.05 statistical difference (ANOVA test) * comparing the groups considering the same treatment and type of sample; # comparing untreated cells with cells treated with melatonin, considering the same group and sample; †comparing between villous and extravillous placental layers considering the same treatment and group.*

TABLE 4 | Viability index (%) and apoptosis (%) of MN cells treated by melatonin from maternal blood and placenta from the non-diabetic (ND) and Type II Diabetes Mellitus (T2DM) groups.

MN cells		ND		T2DM	
		Viable	Apoptosis	Viable	Apoptosis
Maternal blood	MLT (–)	78.9 ± 9.1	21.1 ± 8.5	68.6 ± 12.7	41.4 ± 11.0#
	MLT (+)	80.4 ± 6.8	19.6 ± 5.7	82.9 ± 4.6*	17.1 ± 3.7*
Placenta villous	MLT (–)	76.5 ± 16.4	33.0 ± 5.8	82.5 ± 6.4	16.1 ± 5.7#
	MLT (+)	81.4 ± 9.1	26.1 ± 6.6	78.7 ± 7.0	18.8 ± 5.4#
Placenta extravillous	MLT (–)	79.4 ± 5.1	21.7 ± 11.4†	73.7 ± 11.0	23.8 ± 8.9†
	MLT (+)	78 ± 5.0	22.1 ± 11.4	75.4 ± 10.7	22.8 ± 11.1

The results represent the mean and standard deviation of 10 samples.
MN, Mononuclear cells; MLT, melatonin.
*P < 0.05 statistical difference (ANOVA test) * comparing the groups considering the same treatment and type of sample; # comparing untreated cells with cells treated with melatonin, considering the same group and sample; †comparing between villous and extravillous placental layers considering the same treatment and group.*

Although physiological adaptations occur during pregnancy, hyperglycemia may modify these adaptations and determine a higher frequency of complications in pregnancies of diabetic women (Groen et al., 2015). Furthermore, the higher index of superoxide release can alter intracellular events during oxidative metabolism (Fagundes et al., 2012) and plays a key role in the pathogenesis of diabetes (Fagundes et al., 2018), and these changes may increase apoptosis (França et al., 2016; Honorio-França et al., 2016; Honorio-França et al., 2021). In this work, the increased release of superoxide by MN cells in the maternal blood of hyperglycemic mothers associated with reducing the superoxide dismutase enzyme may be associated with high apoptotic rates.

Apoptosis can be considered an essential physiological mechanism through which undesirable tissues and cells can be programmed to be killed in a controlled and tightly regulated manner (Baskić et al., 2006). These alterations are evidenced through morphological changes with DNA damage (Zimmermann et al., 2001). In hyperglycemic mothers, there is an increase in oxidative stress and DNA damage. The type of affected DNA seems to be dependent on the glycemic profile or oxidative stress because the reactive oxygen species resulting from glucose oxidation are more likely to cause DNA damage (Collins et al., 1998), which would explain the increase in apoptosis in the blood cells of mothers with diabetes. It is worth mentioning the important role of the hormone melatonin in this process since this hormone, due to its antioxidant activity, reduced apoptosis indices in the maternal blood of hyperglycemic mothers.

It is evident that in the placental villous layer of diabetic mothers, there was a reduction of the enzyme superoxide dismutase and the apoptotic rates. One of the processes associated with maternal immune tolerance is the apoptosis of T cells that express the Fas ligand in trophoblasts or decidual cells (Guleria and Sayegh, 2007), which confers immune privilege. The apoptosis of maternal immunologic cells expressing cellular surface receptor Fas (CD95) occurs in the placenta/decidua interface. This receptor can regulate the death of various cell types, including β cells of the pancreas, and is associated with the development of type 2 diabetes (Nolsoe et al., 2006).

A previous study showed that diabetic mothers present lower levels of memory T cells in the placental villous layer, associated with lower expression of Fas, suggesting the commitment of apoptosis in MN cells and, probably, in the immunoregulation of the mother-placenta-fetus unit and maternal–fetal tolerance (Queiroz et al., 2019).

The mechanisms by which melatonin influences the cells seem to involve other hormones, cytokines, and specific receptors (Pandi-Perumal et al., 2008; Morais et al., 2019). Also, melatonin increases umbilical and fetal blood flow and antioxidant capacity

and contributes to the supply of oxygen and nutrients to increase placental efficiency (Lemley and Vonnahme, 2017).

Melatonin plays a vital role in protecting female reproduction. Lower implantation rates, pregnancy deficiencies, higher incidence of menstrual irregularities, infertility, and miscarriage in women are often associated with changes in melatonin levels in normal uterine and placental tissues. This hormone, well established by the pharmaceutical industry, has been used as a drug in treating several diseases and appears to be effective in treating pre-eclampsia. Thus, considering its lack of toxicity, this study corroborates the potential benefits of melatonin in the reproduction of diabetic mothers and may contribute to its future therapeutic use to minimize the pro-oxidative effects caused by hyperglycemia since melatonin acts, in cells and tissues, against oxidative damage potentially improve maternal and neonatal quality of life.

It should be considered that these data were evaluated during a collection period, which can be considered a limitation of this study. Furthermore, it is necessary to continue investigations focusing on other factors and interactions with other hormones or bioactive components that may be important for the fetus-placental unit.

These data suggest that hyperglycemia could alter the balance between superoxide anion production and superoxide dismutase (SOD) enzyme in maternal blood and the placenta of diabetic mothers. These changes reflected in the apoptosis induction mechanisms and were modulated by the hormone melatonin. These results reinforce the importance of the melatonin hormone in the control of oxidative stress and reduction of apoptosis in the maternal blood and the control of apoptosis in trophoblastic cells, which may probably favor maternal–fetal tolerance and the vascularization of the uteroplacental in diabetic mothers.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Research Ethics Committee of School of Medicine Obstetrics Course, UNESP, Botucatu, SP. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ML carried out the assay, participated in the sequence alignment, and drafted the manuscript. DF and AQ participated in collecting samples, carried out the assays, and helped to draft the manuscript. IC participated in the design of the study and helped to draft the manuscript. EF participated in the design of the study and coordination and helped to draft the manuscript. AH-F conceived the study, carried out the assays, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final version of the manuscript.

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Early Abnormal Placentation and Evidence of Vascular Endothelial Growth Factor System Dysregulation at the Feto-Maternal Interface After Periconceptional Alcohol Consumption

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Adequate placentation, placental tissue remodeling and vascularization is essential for the success of gestation and optimal fetal growth. Recently, it was suggested that abnormal placenta induced by maternal alcohol consumption may participate in fetal growth restriction and relevant clinical manifestations of the Fetal Alcohol Spectrum Disorders (FASD). Particularly, periconceptional alcohol consumption up to early gestation can alter placentation and angiogenesis that persists in pregnancy beyond the exposure period. Experimental evidence suggests that abnormal placenta following maternal alcohol intake is associated with insufficient vascularization and defective trophoblast development, growth and function in early gestation. Accumulated data indicate that impaired vascular endothelial growth factor (VEGF) system, including their downstream effectors, the nitric oxide (NO) and metalloproteinases (MMPs), is a pivotal spatio-temporal altered mechanism underlying the early placental vascular alterations induced by maternal alcohol consumption. In this review we propose that the periconceptional alcohol intake up to early organogenesis (first trimester) alters the VEGF-NO-MMPs system in trophoblastic-decidual tissues, generating imbalances in the trophoblastic proliferation/apoptosis, insufficient trophoblastic development, differentiation and migration, deficient labyrinthine vascularization, and uncompleted remodelation and transformation of decidual spiral arterioles. Consequently, abnormal placenta with insufficiency blood perfusion, vasoconstriction and reduced labyrinthine blood exchange can be generated. Herein, we review emerging knowledge of abnormal placenta linked to pregnancy complications and FASD produced by gestational alcohol ingestion and provide evidence of the early abnormal placental angiogenesis-vascularization and growth associated to decidual-trophoblastic dysregulation of VEGF system after periconceptional alcohol consumption up to mid-gestation, in a mouse model.

Keywords: placenta, perigestational alcohol, vascular abnormality, VEGF system, mouse

INTRODUCTION

Normal growth and survival of the fetus depends on the adequate placentation. Besides providing sufficient amounts of nutrients and oxygen, the placenta establishes a privileged immune environment for fetal growth by orchestrating maternal adaptations to pregnancy and acting as a selective and protective barrier to prevent feto-maternal diseases (Creeth and John, 2020). Poor placentation and placental failure compromises fetal development leading to potential chronic diseases in adult (Sharma et al., 2016; Perez-Garcia et al., 2018; Woods et al., 2018). Experimental studies suggest that prenatal alcohol exposure disrupts the placenta (Carter et al., 2016), which may play a crucial etiologic role in alcohol-related fetal effects throughout pregnancy (Hoyme et al., 2016).

Maternal alcohol consumption can lead to the irreversible condition of fetal alcohol syndrome (FAS), the most severe form of the alcohol spectrum disorders (FASDs) (Guerri et al., 2009; de Sanctis et al., 2011; May and Gossage, 2011; Memo et al., 2013; Joya et al., 2015; Van Heertum and Rossi, 2017). FASD is characterized by fetal and birth anomalies, intrauterine growth retardation (IUGR), numerous physical, cognitive, and behavioral defects in newborns and children (Hoyme et al., 2016; Roozen et al., 2016). Prevalence of FASD was estimated at 1–30/1,000 live births in the United States (May et al., 2009; Lange et al., 2017; Popova et al., 2017), and of annual pregnancies, about 40% of women drink some alcoholic beverage during pregnancy while 3–5% of women drink heavily throughout pregnancy (Heller and Burd, 2013). In some Latin-American countries like Uruguay and Argentina, the prevalence of heavy alcohol consumption during pregnancy ranges at 40 to 75% (Magri et al., 2007; López, 2013; López et al., 2015). Despite public efforts to reduce prevalence of alcohol consumption, still high proportion of women often continues to drink moderate levels of alcohol (200 ml/day of wine containing ethanol 11%) during the early pregnancy while unaware they are pregnant (4 to 6 weeks after conception during early organogenesis) (Colvin et al., 2007). In this relation, recently we established a mouse model of perigestational moderate alcohol ingestion, previous and up to early gestation, to study the embryo developmental effects compatible with FASD. Perigestational alcohol intake up to organogenesis (equivalent to the first three-four weeks of human pregnancy) induces delayed embryo differentiation and growth, and dysmorphogenesis, by altering molecular pathways, genotoxicity, apoptosis and oxidative stress (OS) (Cebral et al., 2007, 2011; Coll et al., 2011, 2017). However, despite the direct effects of ethanol exposure on embryo-fetal outcomes, placental injury due to maternal alcohol ingestion was recently proposed as an indirect cause of fetal abnormalities and FASD (Gupta et al., 2016). Maternal alcohol-induced dysfunctional placenta was linked to IUGR, congenital defects, adulthood obesity, metabolic syndromes, cardiovascular disease (Davis et al., 2012a,b; Zhu et al., 2015; Linask and Han, 2016) and fetal programming diseases (Bada et al., 2005; Burd et al., 2007; Gundogan et al., 2008, 2015; Patra et al., 2011; Bosco and Diaz, 2012; Davis-Anderson et al., 2017; Tai et al., 2017). Nevertheless, the etiology of abnormal placenta associated to maternal alcohol consumption is proposed to be related to gestational windows of susceptibility: peri-implantation, gastrulation and/or organogenesis (first trimester in human) (Livy et al., 2004). Both early alcohol and acetaldehyde exposure may contribute to the pathogenesis of FASD by reducing placental growth and function on the first trimester (Lui et al., 2014). Particularly, perigestational moderate alcohol consumption up to peri-implantation prevents blastocyst implantation and results in early pregnancy loss (Perez-Tito et al., 2014). Since maternal alcohol exposure can decrease the trophoblast migration/invasion leading to abnormal placental vascularization (Han et al., 2012), the link between placental vasculopathy and failure in the early decidual-trophoblast development and dysregulation of angiogenesis-vascularization after perigestational alcohol ingestion up to organogenesis recently began to be studied in mouse models (Coll et al., 2018; Ventureira et al., 2019; Gualdoni G. S. et al., 2021).

At present different animal models provide insights into the alcohol-induced mechanisms on the placenta. Nevertheless, the impact of perigestational alcohol ingestion up to early gestation on placental angiogenic mechanisms involved in abnormal placenta has not been sufficiently clarified. Herein, we first provide a brief background on the gestational alcohol placental defects as the etiology of FASD, and then we extend the revision to the knowledge of emerging evidences on the effects of periconceptional consumption up to early organogenesis on placental development, highlighting the role of the trophoblast-decidual VEGF system in a mouse model.

MAIN EFFECTS OF GESTATIONAL ALCOHOL CONSUMPTION ON PLACENTA

Normal placental vascular development is critical for optimal fetal growth, maintenance and successful pregnancy, and subsequent life course (Adamson et al., 2002; Reynolds et al., 2006). The placenta, the major organ determinant of intrauterine growth, is involved in nutrient transport and metabolism of several molecules (Martín-Estal et al., 2019), and in the synthesis and releases of hormones and other mediators into both maternal and fetal circulations (Guttmacher et al., 2014). Alcohol use throughout gestation can disrupt the normal hormonal interactions between mother and fetus, altering natural homeostasis and hence leading to poor pregnancy outcomes. Gestational alcohol exposure impaires the respiratory gases supply due to poor placental vascularity, leading to hypoxia, thus resulting in pregnancy complications, IUGR, and preeclampsia, malnutrition, or stillbirth (Aliyu et al., 2008, 2011; Salihu et al., 2011; Carter et al., 2016). Ethanol interferes with placental transport of nutrients, oxygen, and waste products (Gundogan et al., 2008, 2015; Kwan et al., 2020). Moreover, prenatal alcohol exposure alters the placental iron transport yielding to fetal iron deficiency anemia, condition that exacerbates alcohol-related growth restriction (Kwan et al., 2020). Also, animal and *in vitro* studies have suggested that chronic and heavy alcohol use in pregnancy may impair transport of folic acid across the placenta to the fetus by decreasing expression of transport proteins, thus

contributing to the deficits observed in FASD (Hutson et al., 2012). In overall, alcohol use throughout pregnancy promotes poor fetal outcomes and relevant clinical manifestations of FASD by inducing abnormal placental morphogenesis and impairment of placental metabolism and hormonal function (Gundogan et al., 2008; Ramadoss and Magness, 2012c; Gupta et al., 2016).

Gestational alcohol intake produces the "alcohol-related placental associated syndrome" (Salihu et al., 2011) that includes miscarriage, hypertension, preeclampsia, preterm birth, placenta previa, placenta accreta and placental hemorrhage (Gundogan et al., 2010; Meyer-Leu et al., 2011; Avalos et al., 2014; Carter et al., 2016; Tai et al., 2017; Ohira et al., 2019; Orzabal et al., 2019; Odendaal et al., 2020). Moreover, high risk of placental abruption was observed after consumption of 7–21 drinks per week (a mean of two drinks per day and BAC of 5–100 mg/dL) (Burd et al., 2007). In human and animal models, alcohol exposure during pregnancy usually reduces placental weight and size, affecting directly the structure and function (Gundogan et al., 2008, 2015; Bosco and Diaz, 2012; Carter et al., 2016; Kwan et al., 2020). The most generalized effects of gestational alcohol intake on the placenta were on its vasculature, which is associated with uteroplacental malperfusion, resistance, and placental and umbilical cord contraction (Iveli et al., 2007). In mouse models, alcohol exposure at mid-gestation leads to severe embryo-placental growth retardation (Haycock and Ramsay, 2009).

Gundogan et al. (2008, 2010) reported, in animal model, that one major placental abnormality due to chronic gestational ethanol exposure is the failure of maternal decidual spiral artery remodeling by which the interaction between the invasive trophoblasts and maternal vessels is impared and leads to altered placental blood flow and nutrient exchange. Moderate or high-dose of ethanol intake during gestation also reduces

TABLE 1 | Summary of the main findings on the placental effects produced by gestational alcohol ingestion.

Gestational period of alcohol ingestion	Model	Alcohol intake pattern	Placental effects	References
Along gestation	Human	Moderate quantities	Reduced placental weight	Burd and Hofer, 2008
			Impaired blood flow/artery vasodilatation	Ramadoss and Magness, 2012b,c
			Abnormal nutrient transport	Gundogan et al., 2015
			Fetal resorption, miscarriage	
			Umbilical cord vasoconstriction, IUGR	
Along gestation	Human	High alcohol quantities	Growth restriction, fetal hypoxia	Gundogan et al., 2008, 2010, 2015
			Reduced blood flow and nutrient interchange	
			Fetal hypoxia, IUGR	
Along gestation	Human	Two drinks (wine)/day (BAC 5–100 mg/dL)	Placental abruption, IUGR, FAS	Burd et al., 2007
		(18–30 g ethanol/day)	Abnormal fetus	
Along gestation, or during: 2nd, 3rd, 1st + 3rd or 2nd + 3rd trimesters	Human	Heavy, moderate and/or light drinking	Utero-placental malperfusion and hypoplasia	Tai et al., 2017
			Premature delivery, IUGR	
Gestational days 7–17	Rat	Ethanol 4.5 g/kg/day (BAC 216 mg/dL)	Uterine vascular disfunction	Ramadoss and Magness, 2012c Subramanian et al., 2014
One occasion across gestation	Human	Binge-heavy (8 drinks on 1.5 days/week)	Decreased placental growth	Carter et al., 2016
Along gestation (days 6–18)	Mouse	BAC 110 mg/dL	Placental resistance,	Ramadoss and Magness, 2012a
			Abnormal vascular perfusion	
Gestational days 6–16	Rat	18-24-37% EDC	Incompleted uterine vascular transformation	Gundogan et al., 2008, 2010, 2015
Along gestation	Human	High quantities- severe intake	Vascular resistance, vasoconstriction	Siler-Khodr et al., 2000 Acevedo et al., 2001 Reynolds et al., 2006 Ramadoss and Magness, 2011 Bosco and Diaz, 2012
Chronic binge-like during gestation	Human		Impaired maternal uterine artery reactivity	Subramanian et al., 2014
			Vascular dysfunction	
			Decreased uterine vasodilation	
Pre-conception until early gestational	Primate	1.5 g/kg/day of a 4% ethanol	Reduced vascular perfusion in late placenta	Lo et al., 2017
		(=6 drinks/day)	Altered fetal vasculature in late placenta	
Gestational day 8.75	Mouse	Acute (two i.p injections 3 g/kg ethanol)	Reduced late placental labyrinth	Haghighi Poodeh et al., 2012
			Altered cell junctions of placental barrier	
			Increased permeability	

BAC, blood alcohol concentration; i.p., intraperitoneal; EDC, ethanol derived calories; IUGR, intrauterine growth restriction.

the labyrinthine development (Gundogan et al., 2008, 2010, 2013, 2015). During third trimester of gestation, alcohol affects the uteroplacental vascular function (Rosenberg et al., 2010; Subramanian et al., 2014; Orzabal et al., 2019) by impairment of uterine spiral artery remodeling, angiogenesis and vasodilation (Radek et al., 2005), *via* altered endothelial angiogenic gene expression (Ramadoss and Magness, 2012b) and proteome defects (Ramadoss et al., 2011; Ramadoss and Magness, 2012c). **Table 1** summarized the main relevant findings, on the placental effects produced by gestational alcohol ingestion in human and/or murine models.

Adequate mutual interactions between decidual and trophoblast tissues during early placentation determine the normal vascularization of the placenta at term. Placental defective growth and angiogenesis, linked to altered early maternal vascular remodeling and trophoblast invasion (Woods et al., 2017, 2018), may cause pregnancy failure, placental insufficiency, preeclampsia, fetal developmental disorders, and preterm birth (Cha et al., 2012). Advances in the understanding of placental abnormalities and the main mechanisms involved induced by maternal alcohol exposure are usually studied in the mouse model of placentation (Probyn et al., 2012), which briefly is given below.

OVERVIEW OF MOUSE PLACENTAL DEVELOPMENT AND VASCULARIZATION AS A MODEL FOR PLACENTAL-ALCOHOL EFFECT STUDIES. ROLE OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR SYSTEM

In mouse, placentation begins with implantation (days 4.5–6 of gestation), when blastocyst's mural trophectodermal cells invade the uterine epithelium (Woods et al., 2018). After implantation, placental development goes through the gestational phases of gastrulation (days 6 to 8.5), organogenesis (days 9 to 11.5) and the fetal phase (days 12–19).

Decidual Development and Maternal Vascularization

Decidualization and maternal angiogenesis are pivotal to provide normal placental vascularization at term. Immediately with implantation, antimesometrial uterine stromal cells proliferate and transform in decidual cells, forming the avascular and densely packed decidual tissue (day 6 of gestation). Following decidualization extends toward mesometrial region of the implantation site (days 7.5–10), transformation of uterine spiral arteries occurs by angiogenesis (Das, 2009, 2010). The smooth muscle layer of spiral arteries close to the trophoblastic zone, normally largely remodel and disappear and thus, profound mesometrial decidual vascular growth takes place to allow uterine vascular elongation and provide maternal oxygenated blood flow to the fetal face of placenta (Cross et al., 2002; Kim et al., 2013; Ventureira et al., 2019). Maternal angiogenesis and vasodilation, involving also endothelial proliferation, are promoted mainly by decidual and uterine natural killer cells (uNKs) (Blois et al., 2011; Sojka et al., 2019). The uNKs have vital roles in decidual vascular remodeling and dilation of spiral arteries (Charalambous et al., 2012; Hofmann et al., 2014; Lima et al., 2014), in trophoblast invasion control (Ashkar and Croy, 2001; Ashkar et al., 2003; Croy et al., 2003; Felker and Croy, 2017; Meyer et al., 2017a,b), decidualization, and immune tolerance (Greenwood et al., 2000; Wallace et al., 2012; Rätsep et al., 2015). Mouse strains genetically ablated from uNK cells, not only fail to undergo smooth muscle spiral artery remodeling but also show abnormal branching of the vascular bed, leading to implantation sites with anomalous vascularization (Yougbaré et al., 2017). The VEGF of uNK cells is necessary to guide the maternal angiogenesis (Gargett et al., 2001; Wulff et al., 2002; Zygmunt et al., 2003; Heryanto et al., 2004; Taylor, 2004; Coultas et al., 2005).

Development of Placental-Trophoblast Layer

After implantation, in mouse gestational days 7–8.5, differentiated polyploid invasive primary trophoblast giant cells (TGCs) first invade the maternal microvasculature around the conceptus (Malassine et al., 2003), while the polar trophectoderm forms the extra-embryonic ectoderm and, from it, the chorionic cells and the ectoplacental cone (EPC). The allantois grows out, attaches and fuses to the chorionic trophoblast forming the chorio-allantoic placenta. Meanwhile, secondary TGC cells differentiate at the margin of the EPC, and become invasive to remodel mesometrially the maternal extracellular matrix (ECM) and microvasculature (Woods et al., 2018).

At organogenesis, the mouse placenta consists of three layers: the decidua, the junctional zone (JZ), and the labyrinth. The JZ, the outer layer limiting the decidua, is constituted by spongiotrophoblast cells (SpT), and the invasive cells: TGCs (P-TGC), spiral artery-associated TGCs (SpA-TGCs) and glycogen cells (GC) (Malassine et al., 2003; Woods et al., 2018). The labyrinth develops when the attached allantoic mesoderm invaginates and interdigitates into the chorion, to form the extensive vascular fetal network of definitive placenta. The labyrinth is composed of the fetal vessels, chorionic-mononuclear trophoblast cells and the allantoic mesenchyme. While the fetal vessels undergo extensive branching, the chorionic trophoblasts differentiate into sinusoidal-TGCs and the syncytiotrophoblast cells develop; all togheter form, with the fetal endothelium, the interhemal barrier (Watson and Cross, 2005; Hu and Cross, 2010; Rai and Cross, 2014; Woods et al., 2018).

When the definitive placental vascularization is established (around day 11 of gestation), major trophoblast invasion to maternal spiral arteries begins. While TGCs remodel the decidual ECM (interstitial invasion) (Knöfler et al., 2001; Rai and Cross, 2014), spiral artery-TGCs replace the endothelial cells of dilated spiral arteries (endovascular invasion). This maternal vessel transformation leads to distended, high flow and low-resistance maternal sinusoids at JZ (Cross et al., 2002), where the maternal blood is funneled in trophoblast-lined conduits in absence of arteriolar vasoconstriction. These vascular remodeling processes

directed by trophoblast are major key for successful progression of pregnancy. Abnormal invasion (sub- or over-invasiveness) and maternal endothelial remodeling/replacement are key etiologic mechanisms associated to placental pathologies and pregnancy complications (Perez-Garcia et al., 2018). Placental angiogenesis-vascularization occurs under production and control of growth and vasoactive factors, such as the vascular endothelial growth factor (VEGF) (Hess et al., 2007).

Vascular Endothelial Growth Factor System During Placentation

The VEGF system is involved in angiogenesis-vascularization during placentation (**Figure 1**). VEGF is expressed in arteriolar smooth muscle, endothelium, in trophoblast, uNKs and decidual cells (Hoffmann et al., 2007; Zhang et al., 2011; Hofmann et al., 2014; Li et al., 2014; Ventureira et al., 2019). Its expression is regulated by hypoxia through transcription of the hypoxia inducible factor (HIF)-1α (Maltepe et al., 2005). During early gestation, HIF-1α regulates the trophoblast proliferation and differentiation during EPC development (Takeda et al., 2006). A hypoxic-oxidant environment induces the synthesis and release of anti-angiogenic factors, leading to failures in trophoblastic differentiation, invasion and placental angiogenesis (Perez-Garcia et al., 2018; Woods et al., 2018).

Vascular Endothelial Growth Factor acts through binding to three receptors with intrinsic tyrosine kinase activity: VEGF-R1 (FLT-1), VEGF-R2 (KDR/Flk-1) and VEGF-R3 (FLT-3) (Kimura and Esumi, 2003). Main physiological effects of VEGF are attributed to KDR, whereas FLT-1 modulates VEGF signaling *via* ligand sequestration (Chung and Ferrara, 2011). KDR activation induces cell proliferation and migration

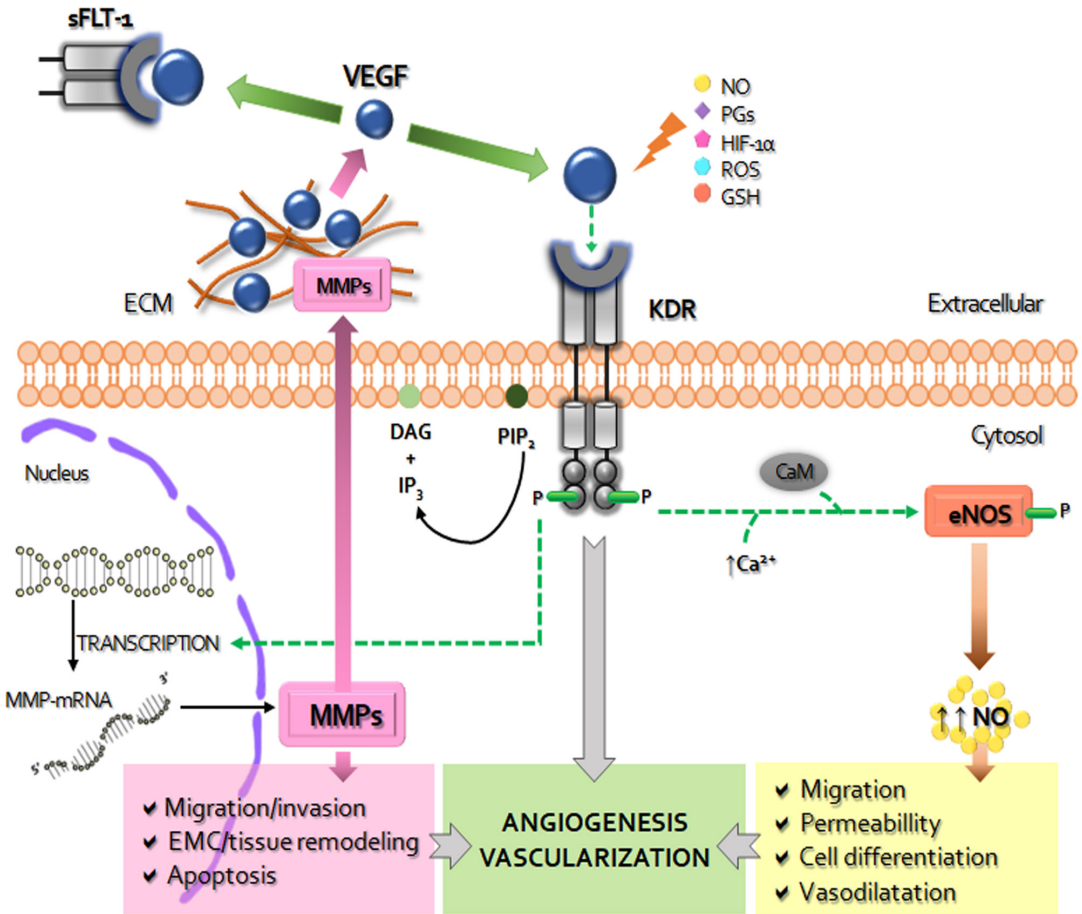

FIGURE 1 | Schematic diagram of the role of VEGF system in the placentation. The vascular endothelial growth factor (VEGF) activates the membrane kinase insert domain receptor (KDR) by phosphorylation, triggering specific signaling cascades. By increasing intracellular calcium (Ca^{++}) and calmodulin (CaM) VEGF-KDR binding induces the expression and activity of nitric oxide synthase (NOS) to produce increase of nitric oxide (NO), a strong inducer of increased vascular permeability, vasodilation, cell migration and differentiation. The other *via* of VEGF-KDR activation, related to fosfatidil inositol 4,5-bisfosfato (PIP-2) and diacylglycerol (DAG), leads to the transcription of metalloproteinases (MMPs). MMPs are secreted to the extracellular matrix (ECM) where, among other functions, degrade and remodel the ECM and release the ECM-sequestered VEGF by cleaving proteins that retain it in the matrix. The soluble receptor tyrosine kinase similar to fms-1 (sFLT-1) can prevent the binding of VEGF to KDR, thus decreasing it pro-angiogenic and survival activity. VEGF expression can be modified by NO, prostaglandins (PGs), hypoxia inducible factor (HIF-1α), reactive oxygen species (ROS), reduced glutathione (GSH), among other factors. In overall, placental activated VEGF system mediates the angiogenesis-vascularization during placentation.

(Lara et al., 2018), permeability (Yancopoulos et al., 2000), decidualization, trophoblastic differentiation and invasion, and nutrient uptake of the placenta (Reynolds and Redmer, 2001; Haghighi Poodeh et al., 2012). In pathological placentas, VEGF is reduced whereas the soluble form of FLT-1, responsible for the endothelial dysfunction, is increased (Roberts and Escudero, 2012; Li et al., 2014).

The VEGF-KDR binding activates signaling cascades that stimulate the production of at least 11 angiogenic factors (Apte et al., 2019), such as the endothelial nitric oxide synthase (eNOS) and matrix metalloproteinases (MMPs). The nitric oxide (NO), produced by oxidation of L-arginine, is an important regulator of the placental vasodilatation, participating in vascular smooth muscle relaxing, the increase of blood flow, and reduction of platelet aggregation and thrombosis (Krause et al., 2011).

The other major contributors to VEGF-mediated angiogenesis are the MMPs. These multigenic proteolytic zinc-dependent enzymes are composed by six classes (collagenases, gelatinases, stromelysins, matrilysins, membrane-type MMPs, and other MMPs) (Amălinei et al., 2007; Cui et al., 2017; Henriet and Emonard, 2019). MMPs are involved in proliferation, apoptosis, migration, differentiation, tissue and ECM remodeling, protein degradation (Lemaître and D'Armiento, 2006; Hamutoğlu et al., 2020), and trophoblast survival and invasion (Isaka et al., 2003; Agaoglu et al., 2016). MMP-2 (gelatinase A) and MMP-9 (gelatinase B) play a role in endometrial tissue remodeling at implantation (Novaro et al., 2002), in decidualization (Fontana et al., 2012), in trophoblast invasiveness (Staun-Ram et al., 2004; Plaks et al., 2013; Espino et al., 2017; Gualdoni G. et al., 2021), in endothelial cell morphogenesis (Chandrasekar et al., 2000). Expansion of the uterus to accommodate the growing embryo and the maternal vascular establishment depend on MMP-2 and -9 (Sternlicht and Werb, 2001). At early mouse gestation, TGCs are positive for MMP-2 and MMP-9 expression (Alexander et al., 1996; Bany et al., 2000; Bai et al., 2005), while during organogenesis mainly MMP-9 participates in TGC-invasion and labyrinthine vascularization (Fontana et al., 2012; Gualdoni G. et al., 2021; Gualdoni G. S. et al., 2021). VEGF up-regulates the MMP-2 and MMP-9 expression in human umbilical vein endothelial cells (Heo et al., 2010). Imbalances of VEGF system and subsequent alterations in MMP-2 and MMP-9 expression-activity take relevance in abnormal placentation (Gualdoni G. S. et al., 2021) and in various placentopathies (Amălinei et al., 2007; Cui et al., 2017).

PATHOPHYSIOLOGIC MECHANISMS INVOLVED IN ALCOHOL-ASSOCIATED PLACENTAL ABNORMALITIES AND ROLE OF THE ANGIOGENIC VASCULAR ENDOTHELIAL GROWTH FACTOR SYSTEM

The alcohol-induced abnormal placental mechanisms are multifactorial. The alcohol dispersed into placenta is primary detoxified by other placental systems (CYP2E1) different from the alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes (Gemma et al., 2007), because of their low affinity/activity in placental tissues (Heller and Burd, 2013). By this, gestational alcohol exposure impacts on the metabolism of placenta producing oxidative stress (OS) (Kay et al., 2000; Gundogan et al., 2008, 2010, 2015). Although antioxidant enzymatic and glutathione activities have been shown in the placenta after gestational alcohol ingestion (Qanungo and Mukherjea, 2000), OS impacts strongly on trophoblastic function and leads to pregnancy loss (Gundogan et al., 2010). Perigestational moderate alcohol ingestion up to organogenesis in mouse produces protein nitration, lipid peroxidation and DNA damage in trophoblast-decidual tissue (Coll et al., 2018). As a result of alcohol-induced OS and decreased bioavailability of NO, insufficient early placental vascularization and arteriolar vasoconstriction can cause placental hypoxia (Acevedo et al., 2001; Wareing et al., 2006; Gualdoni G. S. et al., 2021).

Stimulatory and inhibitory effects of ethanol on VEGF have been reported, depending on alcohol administration patterns (Radek et al., 2008; Haghighi Poodeh et al., 2012; Jegou et al., 2012). In chick extraembryonic tissues, moderate and heavy alcohol exposure for 24 or 48 h, impaires vascular development and downregulates VEGF and its receptors (Gu et al., 2001; Tufan and Satiroglu-Tufan, 2003). Together with enhanced permeability and altered placental barrier, VEGF was up-regulated in the CD-1 mouse placenta at 9.5–14.5 days of gestation after two 4-h interval intraperitoneal doses of 3 g/kg ethanol injected 8.75 days post-coitum (Haghighi Poodeh et al., 2012). Ethanol treatment decreases VEGF in yolk sac membranes by inhibition of angiogenic genes due to excess of alcohol-induced reactive oxygen species production (Wang et al., 2016). However, diminished placental vascular density after early alcohol exposure significantly decreased KDR expression in placenta at term (Holbrook et al., 2019). In addition, in alcohol-induced hypoxic placenta, the release of anti-angiogenic soluble receptor sFlt-1 (Reyes et al., 2012), is associated with maternal endothelial dysfunction (Roberts and Cooper, 2001; Roberts et al., 2011). Thus, alterations in the VEGF-VEGF-R, caused by oxidative stress, may be the main cause important imbalances in placental angiogenesis induced by alcohol.

The VEGF downstream molecular expression, such as NO and MMPs, may be altered by alcohol. Chronic binge-like alcohol decreases uterine arterial endothelial eNOS expression in an animal model of third trimester-equivalent of human pregnancy (Ramadoss et al., 2011), in a similar way as does the acute ethanol exposure in human placenta. However, chronic and acute ethanol exposure seems to induce eNOS activity in the fetoplacental unit in other model and HUVEC cells, respectively (Acevedo et al., 2001). Anyway, inhibition or stimulation of NO synthesis by alcohol use throughout pregnancy leads to vasoconstriction of the placenta and umbilical vessels and results in hypoxia and reduced fetal malnutrition (Lui et al., 2014; Gundogan et al., 2015; Lo et al., 2017; Holbrook et al., 2019; Ohira et al., 2019). Consequently, the impaired placental function leads to an increase in oxidative stress that compromises placentation as it alters trophoblast cell motility (Kay et al., 2000; Gundogan et al., 2008). Also, gestational

alcohol exposure can alter transcription of MMPs and affects the maternal uterine vascular remodeling (Orzabal et al., 2019).

PERIGESTATIONAL ALCOHOL CONSUMPTION UP TO EARLY GESTATION: EFFECTS ON MOUSE PLACENTATION AND ROLE OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR SYSTEM

The origins of uteroplacental insufficiency and vasculopathy at late gestation may be caused by abnormal placentation during early pregnancy, including the peri-implantation period (Burton and Jauniaux, 2010; Kajantie et al., 2010). Evidence is currently lacking, in animal models, to explain the effects and cellular-molecular pathways responsible for late placental abnormalities induced by perigestational alcohol consumption (PAC) until early gestation (Gårdebjer et al., 2014; Kalisch-Smith and Moritz, 2017; Kalisch-Smith et al., 2019). Here, in a mouse model, we propose that PAC up to organogenesis disrupts the early decidual-trophoblastic development and vascularization (**Figures 2A,B**; Perez-Tito et al., 2014; Coll et al., 2018; Ventureira et al., 2019; Gualdoni G. S. et al., 2021), leading to incomplete maternal vascular remodeling due to trophoblast invasion defects (**Figure 2C**), and later, to abnormal placenta (**Figure 2D**).

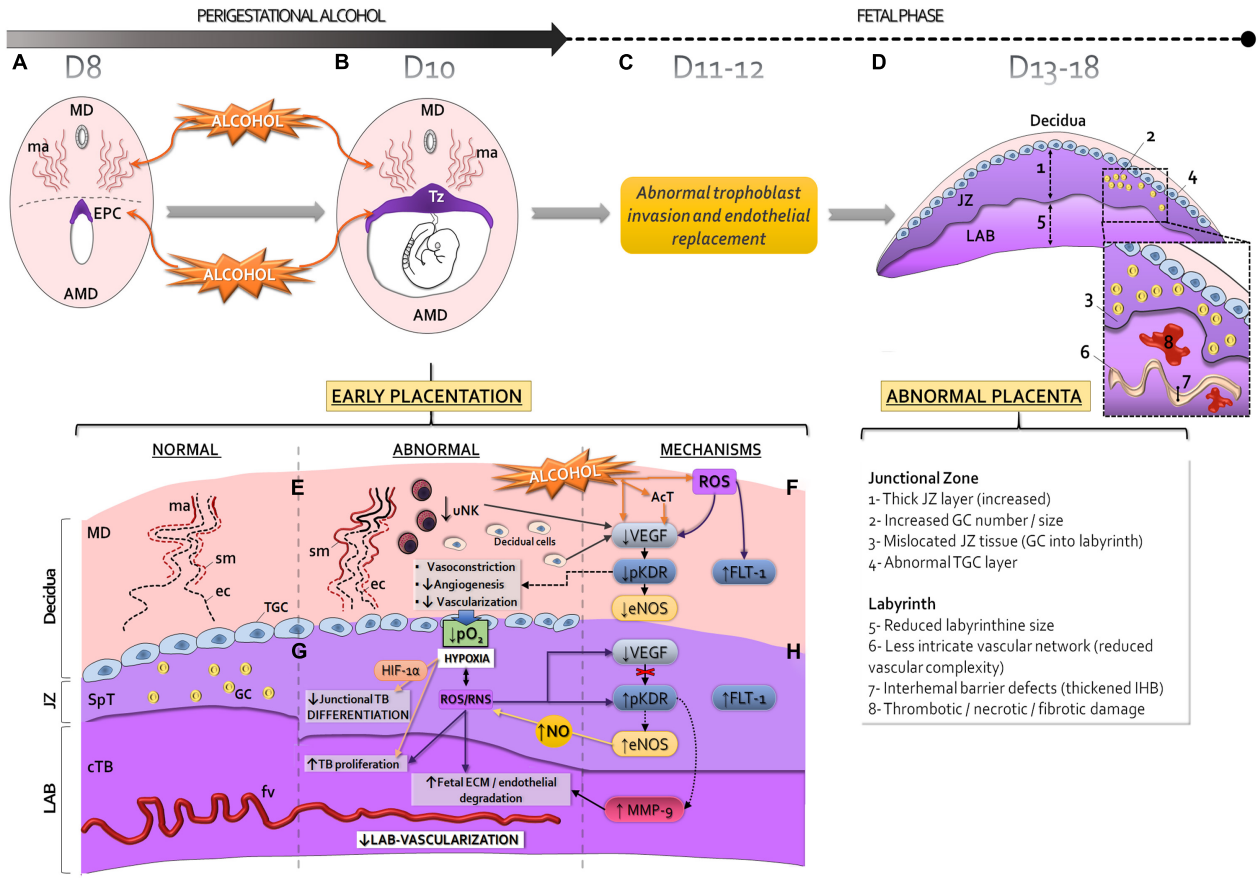

FIGURE 2 | Proposed mechanisms of abnormal early placentation and vascularization and long-term impact to placenta produced by perigestational alcohol consumption, in the mouse model. Perigestational alcohol (OH) intake up to gastrulation-organogenesis [day 8 (D8) **(A)** and 10 (D10) **(B)**, disrupts the maternal spiral artery (ma) remodeling of mesometrial decidua (MD), and alters the ectoplacental cone (EPC) **(A)** and subsequent development of trophoblastic zone (Tz) of the early placenta **(B)**. Consequently, after cessation of alcohol ingestion (day 11, D11), abnormal trophoblast invasion and endothelial replacement may occur **(C)**, leading to abnormal structure and function of the placenta at term **(D)**. The placental abnormalities can be originated in altered angiogenesis-vascularization of decidual-trophoblastic tissues **(E,G)** during perigestational alcohol exposure **(B)**. Alcohol and/or its metabolites [acetaldehyde, AcT, reactive oxygen species (ROS)] impact on the early placentation processes, including changes in uNK cells. Consequently, diminished junctional trophoblast differentiation and low labyrinthine growth and vascularization can occur **(G)**. The decidual-trophoblastic abnormalities can be associated with altered endothelial nitric oxide synthase (eNOS) and metalloproteinases (MMP)-2 and/or MMP-9 expression/activity due to abnormal vascular endothelial growth factor (VEGF) expression and KDR activation (pKDR) or increased FLT-1 **(F,H)**. The disrupted VEGF-receptor-NOS-MMPs mechanisms are linked to hypoxia and increased HIF-1α, and reactive oxygen species and/or reactive nitrogen species (ROS/RNS) production. Sm, smooth muscle cells of spiral arteries; ec, endothelial cells; cTB: chorionic trophoblastic cells; SpT, spongiotrophoblastic cells; GC, glycogen cells; TGC, trophoblast giant cells; fv, fetal vessels; Lab, labyrinth; JZ, junctional zone; TB, trophoblast; ECM, extracellular matrix; AMD, antimesometrial decidua.

After PAC up to day 10 of gestation, the lumen expansion of decidual spiral arteries is reduced (**Figure 2E**; Ventureira et al., 2019), producing a poor dilation of maternal vascular bed. Alcohol could alter the decidual artery endothelial organization and reduces cell proliferation. PAC disrupts the arterial smooth muscle cell remodeling and leads to permanent muscle wall in decidual vessels. These defects could be associated to decreased number of uNK cells in decidua, which may also be involved in low dilation and less branching of maternal spiral arteries (**Figure 2E**; Ventureira et al., 2019).

Reduced decidual angiogenesis after PAC can be explained in part by the decreased VEGF expression in decidual and uNK cells probably due to OS (Coll et al., 2018; Ventureira et al., 2019; **Figure 2F**). However, down-regulation of KDR expression in decidual and endothelial cells after PAC could also be involved in the abnormal decidual vascularization. FLT-1 drives anti-angiogenic effects by its binding to VEGF (Ferrara, 2004; Lima et al., 2014; Felker and Croy, 2017), and its increase was associated with oxidative factors (Kim et al., 2013). After PAC, OS may induce FLT-1 expression in decidual and uNK cells causing abnormal decidual angiogenesis (Ventureira et al., 2019; **Figure 2F**).

Impaired downstream VEGF signaling, due to reduced activation of KDR (pKDR) after alcohol consumption, leads to a decreased expression/activity of eNOS in the decidual endothelium, contributing to maternal artery vasoconstriction and reduced angiogenesis (**Figure 2F**). The immediate consequence of low decidual vascularization, because of unremodeled maternal vessels, is a deficient blood perfusion, increased blood pressure and flow velocity. Reduced tissue oxygenation increases HIF-1α expression levels in the trophoblastic interface, which is consistent with a hypoxic-oxidative state (Gualdoni G. S. et al., 2021; **Figure 2G**).

At organogenesis, the labyrinth is still growing in a low oxygen environment, but junctional trophoblastic cells become more differentiated and invasive with increased oxygenation (Cowden Dahl et al., 2005; Pringle et al., 2010). PAC produces trophoblastic growth deficiency and TGC and spongiotrophoblast cell abnormalities compatible with trophoblast differentiation alterations (Gualdoni G. S. et al., 2021; **Figure 2G**). Subsequent abnormal invasion (**Figure 2C**) can lead to placentopathy later (Woods et al., 2017; **Figure 2D**). Paralely, the PAC-induced deficient labyrinthine vasculogenesis is associated to a densely packed tissue due to increased chorionic trophoblastic cell proliferation (Gualdoni G. S. et al., 2021; **Figure 2G**). The early insufficient labyrinthine vascularization generates persistent hypoxia and OS and embryo growth restriction and malformations at organogenesis (Cebral et al., 2007; Coll et al., 2011, 2017; Gualdoni G. S. et al., 2021).

Although hypoxia is a strong stimulus for placental VEGF expression (Zhang et al., 2015), this expression decreased in the exposed-trophoblastic tissues (Gualdoni G. S. et al., 2021). Despite VEGF reduction, probably due to OS and/or to sequestering by FLT-1, alcohol induces high trophoblastic KDR phosphorylation (**Figure 2H**). Following PAC, downstream KDR activation results in increased trophoblastic eNOS expression, which over-produces NO, causing placental OS. However, eNOS expression could also be triggered by FLT-1 pathways (Bussolati et al., 2001) or induced by hypoxia since eNOS promoter contains hypoxia response elements (Schäffer et al., 2006). Once ethanol induces NO production, this factor is able to cause phosphorylation of KDR (Bussolati et al., 2001). Despite KDR activation in JZ and labyrinth, MMP-2 and MMP-9 expression decreased in the former but only MMP-9 increased in the later, resulting in tissue-dependent adverse alcohol effects in the exposed trophoblastic tissues (Gualdoni G. S. et al., 2021; **Figure 2H**).

In conclusion, PAC up to organogenesis leads to early abnormal placentation by defective decidual-trophoblastic development and disruption of complex angiogenic cellular processes, in which the VEGF system results one of the major affected mechanisms. PAC up to early pregnancy may lead to placental abnormalities and vasculopathy compatible with abnormal placentas associated to FASD.

AUTHOR CONTRIBUTIONS

EC have proposed the topic of this revision and prepared the draft of the manuscript. GG and EC edited the text. GG designed the figures. PJ, CB, and MV co-wrote the manuscript. All authors approved the last version of the manuscript.

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How Soluble Fms-Like Tyrosine Kinase 1 Could Contribute to Blood-Brain Barrier Dysfunction in Preeclampsia?

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Preeclampsia is a pregnancy-related syndrome that courses with severe cerebrovascular complications if not properly managed. Findings from pre-clinical and clinical studies have proposed that the imbalance between pro- and anti-angiogenic factors exhibited in preeclampsia is a major component of its pathophysiology. In this regard, measurement of circulating levels of soluble tyrosine kinase-1 similar to fms (sFlt-1), a decoy receptor for vascular endothelial growth factor (VEGF), is a moderately reliable biomarker for the diagnosis of preeclampsia. However, few studies have established a mechanistic approach to determine how the high levels of sFlt-1 are responsible for the endothelial dysfunction, and even less is known about its effects at the blood-brain barrier (BBB). Since the expression pattern of VEGF receptors type 1 and 2 in brain endothelial cells differs from the observed in peripheral endothelial cells, and components of the neurovascular unit of the BBB provide paracrine secretion of VEGF, this compartmentalization of VEGF signaling could help to see in a different viewpoint the role of sFlt-1 in the development of endothelial dysfunction. In this article, we provide a hypothesis of how sFlt-1 could eventually be a protective factor for brain endothelial cells of the BBB under preeclampsia.

Keywords: cerebrovascular complications, blood-brain barrier, stroke, VEGF, sFlt-1, preeclampsia

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INTRODUCTION

According to the International Society for Study of Hypertension in Preeclampsia (ISSHP), preeclampsia is defined as the presence of *de novo* hypertension (≥140/90 mmHg) after 20 weeks of gestation accompanied by proteinuria and/or a series of systemic symptoms and/or fetal growth restriction (Brown et al., 2018). Preeclampsia affect 1–5% of pregnancies, and it would be responsible for 70,000–80,000 maternal deaths and 500,000 perinatal deaths worldwide every year (Hutcheon et al., 2011). In Latin America and the Caribbean, hypertensive disorders account for almost 26% of maternal deaths (Khan et al., 2006). Causes of maternal death due to preeclampsia in low- and middle-income countries are mainly associated with cerebrovascular complications (Miller, 2019).

In human, preeclampsia is a pregnancy-related syndrome that requires the presence of a placenta to develop (Redman and Sargent, 2005; Kanter et al., 2010). The two-stage model proposes a poor placentation leads to the placental release of harmful factors into the maternal circulation, resulting in the development of endothelial dysfunction (Roberts et al., 1989; Redman and Sargent, 2005). Under this sequence of events, the cerebrovascular complications of preeclampsia represent an additional example of multisystemic endothelial damage. This model has evolved over the years including factors such as inflammation, angiogenic imbalance and maternal risk factors (Staff, 2019). However, this point of view still seems to be an oversimplification of the pathogenesis of cerebral complications, as approximately one third of the women with eclampsia develop mild hypertension (Sibai, 2012) and cerebral complications are also detected post-partum (Razmara et al., 2014).

Among the circulating placental factors present in the maternal circulation, the soluble truncated vascular endothelial growth factor (VEGF) receptor type 1 or tyrosine kinase-1 similar to fms (sFlt-1), has received increasing attention due to its clinical applicability as risk factor for preeclampsia (Maynard et al., 2003; Levine et al., 2004). High levels of sFlt-1 are thought to lead to an angiogenic imbalance, but despite hundreds of publications focusing on sFlt-1 and preeclampsia, the underlying cellular mechanism by which sFlt-1 generate endothelial dysfunction in this syndrome are poorly known, and even less is known about its potential effects at the blood-brain barrier (BBB).

Physiologically, VEGF binds and activates the tyrosine-kinase receptors VEGF receptor type 1 (VEGFR1), type 2 (VEGFR2), and type 3 (VEGFR3) (Simons et al., 2016). Since sFlt-1 works as a decoy VEGFR with greater binding affinity to VEGF compared to VEGFR2, this function balances the effects of VEGF signaling on vascular development, permeability, and integrity. Therefore, sFlt-1 is an antagonist to VEGFR2 signaling.

Vascular endothelial growth factor receptor type 2 is predominately expressed in vascular endothelial cells, including brain endothelial cells, where activation of VEGFR2 consistently leads to increased permeability of the BBB (Schreurs et al., 2012; Hudson et al., 2014), as observed in pathological conditions including stroke (Li et al., 2014) and multiple sclerosis (Argaw et al., 2012; Chapouly et al., 2015). Several reports have demonstrated that brain endothelial cells become more reactive to the paracrine secretion of VEGF from neurons and astrocytes under pro-inflammatory and hypoxic conditions (Argaw et al., 2012; Li et al., 2014; Chapouly et al., 2015), being this outcome consistent with the higher basolateral expression of VEGFR2 in this cell type, when compared to the observed in peripheral endothelial cells (Hudson et al., 2014). This compartmentalization of VEGF signaling in brain endothelial cells invites to reassess the role of sFlt-1 in the development of brain endothelial dysfunction in preeclampsia. In this article, we provide a hypothesis of how sFlt-1 could eventually be a protective instead of harmful factor for brain endothelial cells of the BBB, and how it may contribute to the pathophysiology of cerebrovascular complications in preeclampsia based on the current state of the art.

CEREBROVASCULAR COMPLICATIONS IN PREECLAMPSIA

Preeclampsia-associated cerebrovascular complications include eclampsia (new onset of seizures in women with preeclampsia), haemorrhagic and ischemic stroke, edema formation, brain herniation, posterior reversible encephalopathy syndrome (PRES), and reversible cerebral vasoconstriction syndrome (RCVS) (Hammer and Cipolla, 2015). These alterations may cause short- and long-term morbidities including white matter injuries (Hecht et al., 2017; Siepmann et al., 2017), higher post-partum cerebrovascular disease risk and maternal death. Cerebrovascular alterations are the direct cause of approximately 40% of maternal deaths (MacKay et al., 2001).

Epidemiologically, the estimated prevalence of stroke during pregnancy and post-partum is 34 per 100,000 deliveries (James et al., 2005), and a study reported that the risk of post-partum stroke within 60 days after delivery in women with pregnancy-related hypertension is 41.7% (Too et al., 2018). However, the exact prevalence of PRES in preeclamptic and eclamptic women is not well known, but a retrospective study found that PRES was present in more than 90% of eclamptic women and about 20% of preeclamptic women with neurological symptoms (Liman et al., 2012).

Regarding the long-term complications of preeclampsia, some authors have agreed that the white matter injuries observed on magnetic resonance imaging (MRI) several years after delivery, could be related to a higher risk of dementia or stroke (Aukes et al., 2012; Wiegman et al., 2014; Siepmann et al., 2017).

THE BLOOD-BRAIN BARRIER IN PREECLAMPSIA

The BBB is a neurovascular unit that separates the brain tissue from systemic circulation. The brain capillary endothelial cells are the main element that forms the basic unit of BBB, along with the astrocytes, pericytes, and the adjacent neurons (Cantrill et al., 2012; Daneman and Prat, 2015). The endothelial cells join through strong tight junction proteins and substance transport is highly restricted due to the above feature and expression of membrane transporters belonging to the ATP-binding cassette and Solute Carrier families (Qosa et al., 2015; Morris et al., 2017).

Authors have proposed that the increased BBB permeability in preeclamptic pregnancies may be due to: (i) elevated microvascular pressure leading to vasogenic edema formation, (ii) alterations in expression/function of tight junction proteins, and (iii) circulating factors that increase the BBB permeability (i.e., altering transcellular transport) without modifying the mechanical barrier properties (Cipolla and Kraig, 2011).

To characterize the mechanisms involved in the cerebrovascular complications of preeclampsia, animal models have been vastly employed (Li et al., 2012; Warrington et al., 2014, 2015; Zhang and Warrington, 2016). However, in human, most of the research aiming to study the changes in the BBB functionality of preeclamptic women is carried out through MRI studies (Schwartz et al., 2000) and measurement of circulating

levels of biomarkers of brain injury and endothelial damage (Bergman and Akerud, 2016; Bergman et al., 2016, 2018). Findings from these studies generally agree that preeclamptic women may suffer some degree of cerebrovascular damage. Recent studies published by our group showed that exposure of hCMEC/d3 cell monolayers (a human brain endothelial cell line) to plasma from preeclamptic pregnancies increased permeability to FITC-dextran 70 kDa and reduced their transendothelial electrical resistance (Bergman et al., 2021; Leon et al., 2021), with no changes in tight junction proteins mRNA expression (Bergman et al., 2021).

OVERVIEW OF VEGF SIGNALING AND ITS ROLE ON THE CEREBROVASCULAR COMPLICATIONS OF PREECLAMPSIA

The VEGFs family is comprised of homodimeric proteins present in five different isoforms: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF) (Simons et al., 2016). These proteins, also known as angiogenic factors, are involved in vasculogenesis, vascular permeability (including brain circulation), nitric oxide synthesis stimulation, and endothelial cell survival. PlGF regulates the endothelial cell viability and the angiogenesis through its signaling pathways or amplifying VEGF-mediated actions (Schreurs et al., 2012).

The human VEGF receptors (VEGFRs) are tyrosine-kinase transmembrane receptors. Members of this family include VEGFR1 or Flt-1, VEGFR2 or KDR, and VEGFR3 or Flt-3. VEGFR2 have a higher tyrosine-kinase effect than Flt-1 and is the most relevant receptor regarding permeability and growth effects. VEGFR3 seems to participate in vascular development, but its primary function resides in the lymphatic vessels (Simons et al., 2016). VEGF presents an affinity for both, Flt-1, and VEGFR2 receptors, while PlGF binds only to Flt-1 (Schreurs et al., 2012). The soluble fraction of Flt-1 that contains the extracellular-ligand binding sites but lacks the intracellular and transmembrane sites, works as a decoy receptor that sequestrates VEGF and forms a heterodimer with VEGFR2, blocking the activation of the latter (Palmer et al., 2017).

Weeks before the onset of preeclampsia, the placenta secretes sFlt-1 (Levine et al., 2004; Chaiworapongsa et al., 2005). This outcome is thought to explain the elevated levels of this anti-angiogenic factor in the blood of preeclamptic women. sFlt-antagonize both VEGF and PlGF effects by reducing the circulating levels of the active forms, contributing to the clinical manifestations in preeclampsia (Maynard et al., 2003). In pregnant normotensive women there is a physiological increase in sFlt-1circulating levels and a reduction of PlGF between 33 and 36 weeks (Taylor et al., 2003), but in preeclampsia there is a loss of the balance between pro-angiogenic and anti-angiogenic factors that lead to the release of mediators from the placenta to the maternal circulation. This form of communication between the placenta and the maternal circulatory system is proposed to be responsible for the disruption of BBB, and the available evidence implies that not only sFlt-1 but VEGF, PlGF, pro-inflammatory cytokines and extracellular vesicles are also involved (Schreurs et al., 2012; Warrington et al., 2015; Clayton et al., 2018; Leon et al., 2021).

In preeclampsia, the imbalance between angiogenic and anti-angiogenic factors is proposed to play a key role in the increased cerebrovascular permeability (Bean et al., 2018). However, the exact mechanism by which the preeclampsia-mediated alterations in VEGF signaling modify the integrity of the BBB remain poorly known. Physiologically, it is well-documented that VEGFs cause transient opening of endothelial cell-cell junctions and VEGFR2 seems to be the main regulator of cell permeability. Some of the elucidated pathways for the increased permeability involve VEGFR2 signaling toward the Rous sarcoma homology 2-domain (Src) mediated by the T cell-specific adaptor (TSAd) (Sun et al., 2012), eNOS/NO mediated permeability (Fukumura et al., 2001), and caveolin-1 regulation of VEGF-signaling (Lin et al., 2007).

In the context of preeclampsia, a study demonstrated that plasma from preeclamptic pregnancies increased the permeability of cerebral rat veins (Amburgey et al., 2010), being this effect counteracted by co-treatment with a VEGFR2 inhibitor. The above outcome is confirmed in human hCMEC/d3 cells, as plasma from preeclamptic pregnancies, apart from increasing the permeability, up-regulated the mRNA expression of VEGFR2 and phosphorylation at the tyrosine residue Y951 (pY951), along with a decreased phosphorylation at the Y1175 (pY1175) residue (Bergman et al., 2021). The above findings agree with those that relate the pY951 with increased endothelial permeability (Matsumoto et al., 2005).

IS THE ENDOTHELIAL DYSFUNCTION AT THE BBB IN PREECLAMPSIA A RESULT OF HIGH sFlt-1 LEVELS?

It is well-known that preeclamptic women exhibit higher sFlt-1 levels, and this outcome is correlated to endothelial dysfunction (Maynard et al., 2003). The seminal work of Maynard et al. (2003), in which pregnant and non-pregnant mice injected with an adenovirus expressing sFlt-1, demonstrated that high levels of this protein are sufficient to elicit hypertension and glomerular endotheliosis. Further studies proposed a vicious cycle between sFlt-1, hypoxia and oxidative stress in the context of preeclampsia (Karumanchi and Bdolah, 2004). For example, mice exposed to exogenous sFlt-1 showed hypoxia and oxidative stress in the trophoblast, which caused more secretion of sFlt-1 (Jiang et al., 2015). A recent report shown that HUVECs treated with sFlt-1 exerted a pro-apoptotic effect by triggering the mitochondrial apoptosis pathway (Zhai et al., 2020). This hypothesis of the pathophysiology of preeclampsia has led to the development of strategies to restore the angiogenic balance. The removal of sFlt-1 from the circulation by apheresis has shown promising results as a potential treatment of preeclampsia, since increases the levels of free VEGF and PlGF in serum samples of preeclamptic patients (Matin et al., 2020).

However, it must be stressed that sFlt-1 is not the sole responsible of the anti-angiogenic imbalance in preeclampsia. Soluble endoglin (sEng), an anti-angiogenic protein that acts

inhibiting the activity of transforming growth factor β1 (TGFβ1) (Levine et al., 2006), is also a contributing mediator. Studies have proposed that high levels are related to the severity of this syndrome as observed in women developing hemolysis elevated liver enzymes low platelets (HELLP) syndrome (Venkatesha et al., 2006), late-onset preeclampsia (Cim et al., 2017), and animal models of HELLP (Bean et al., 2018). TGFβ1 appears to modulate VEGFR2 signaling in endothelial cells, which results on the loss of both tip cell and stalk cell phenotypes (Jarad et al., 2017). However, its role on permeability needs to be further investigated.

The above findings are consistent with reports demonstrating that treatment of HUVECs with plasma from preeclamptic women causes morphological changes, reduces cell proliferation, elicits mitochondrial damage, increases the permeability, and promotes apoptosis (Wu et al., 2012; Gao et al., 2016). A graphic description of the angiogenic imbalance in preeclampsia is presented in **Figure 1**.

According to the presented evidence, it appears that sFlt-1 can exert endothelial dysfunction by itself or in combination with sEng. However, as preeclampsia can lead to cerebrovascular complications without proper management, a group of studies conducted in rodent models attempted to characterize the effects of sFlt-1 in the brain vasculature. Surprisingly, a report demonstrated that overexpression of sFlt-1 in adult mice does not elicit significant changes in BBB permeability (Maharaj et al., 2008). However, mice overexpressing sFlt-1 and sEng exhibited increased BBB permeability (Maharaj et al., 2008). In a model of HELLP syndrome, induced by chronic administration of sFlt-1 and sEng to pregnant mice, a regional increase in BBB permeability at the posterior cortex was observed (Bean et al., 2016, 2018). Since the administration of exogenous sFlt-1/sEng leads to the development of a HELLP-like syndrome, other models such as the reduced utero-placental perfusion (RUPP) (Li et al., 2012) could be more suitable to study the cerebrovascular complications of preeclampsia. The RUPP model increases sFlt-1/sEng levels (Gilbert et al., 2007, 2009; Sun et al., 2020; Saif et al., 2021) and the permeability of the BBB (Warrington et al., 2014), but none of the presented evidence has proposed a mechanism by which these anti-angiogenic factors could be involved in the cerebrovascular complications elicited by this condition.

The above findings reinforce the hypothesis that the anti-angiogenic imbalance in preeclampsia has a peripheral source and requires a combination of factors to induce endothelial dysfunction at the BBB. However, in other pathological conditions involving the participation of VEGF signaling, the outcomes are contrasting. For example, in stroke (also a cerebrovascular complication of preeclampsia) the acute release of VEGF is known to increase BBB permeability

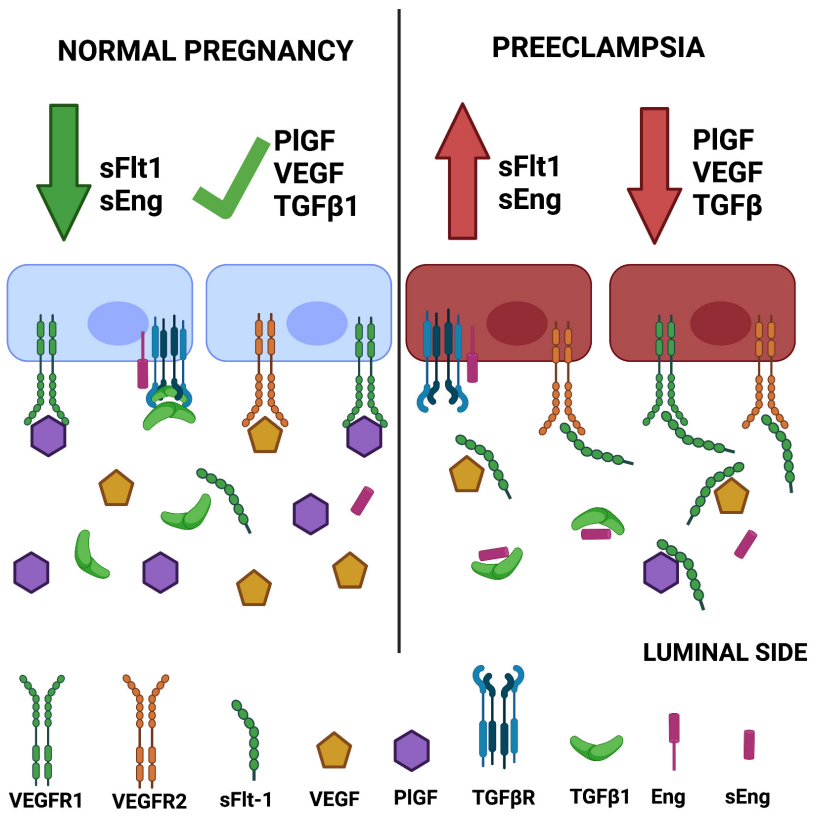

FIGURE 1 | Angiogenic imbalance in preeclampsia. The classic hypothesis of angiogenic imbalance proposes that circulating levels of soluble tyrosine kinase-1 similar to fms (sFlt-1) and soluble endoglin (sEng) are increased in preeclampsia, reducing the concentrations of free vascular endothelial growth factor (VEGF)/placental growth factor (PlGF) and TGFβ, respectively. This outcome leads to endothelial dysfunction. Created with BioRender.com.

(Geiseler and Morland, 2018). A study conducted in patients with subarachnoid hemorrhage demonstrated that under delayed tissue ischemia, the levels of VEGF and sFlt-1 increase in plasma/serum and cerebrospinal fluid (CSF) (Scheufler et al., 2003). The authors suggested that the source of higher VEGF and sFlt-1 levels in *cis*ternal CSF and systemic circulation comes from the local release from endothelial cells and glioneuronal secretion, and thrombocyte aggregations in ischemic brain regions, respectively. A recent study from our group demonstrated that patients with a first-ever ischemic stroke exhibited high circulating levels of VEGF, shortly after the ischemic event. This outcome is associated with a poor prognosis at the sixth month (Escudero et al., 2021).

In rodent models of acute brain ischemia/reperfusion, the gene transfer of sFlt-1 into the lateral ventricle reduced infarction, edema and BBB permeability (Kumai et al., 2007). The authors suggested that the acute effects of VEGF in BBB permeability were attenuated through reduction in the phosphorylation of focal adhesion kinase but did not provided findings to support this hypothesis. This protective effect of sFlt-1 on VEGF-induced BBB permeability has also been demonstrated in cerebral rat veins co-treated with exogenous sFlt-1 and plasma from late-pregnant rats, which exhibit higher levels of sFlt-1 (Schreurs et al., 2012).

According to the presented evidence, sFlt-1 exerts deleterious or protective effects on the brain vasculature depending on the type of insult applied. In preeclampsia, the hypothesis of endothelial dysfunction mediated by an imbalance between pro- and anti-angiogenic factors is often discussed assuming that in pregnancy, BBB endothelial cells can adapt to changes in blood VEGF levels through an increase in sFlt-1 as compensatory mechanism. However, this assumption can be misleading as in murine brain endothelial cells, the expression of VEGFR2 is polarized with higher expression in the basolateral side, while VEGFR1 is more expressed in the luminal side, contrary to the observed in lung endothelial cells (Hudson et al., 2014). Furthermore, this expression pattern is reflected by the formation of homodimers of each receptor, and presence of VEGFR1/2 heterodimers could be a result of the expression of residual basal VEGFR1 and luminal VEGFR2 (Hudson et al., 2014).

This compartmentalization of VEGF signaling BBB endothelial cells is important to understand their reactivity as they are exposed to autocrine and paracrine secretion of VEGF from neurons and astrocytes (Ogunshola et al., 2002), especially under hypoxic and inflammatory conditions (Liu et al., 2020) as observed in pathological conditions such as stroke (Li et al., 2014) and multiple sclerosis (Argaw et al., 2012; Chapouly et al., 2015). For instance, under hypoxic conditions, the release of VEGF in brain cells is regulated by the hypoxia inducible factor-1α (HIF-1), since the *VEGF* gene is activated by this transcription factor (Hu et al., 2003). Furthermore,

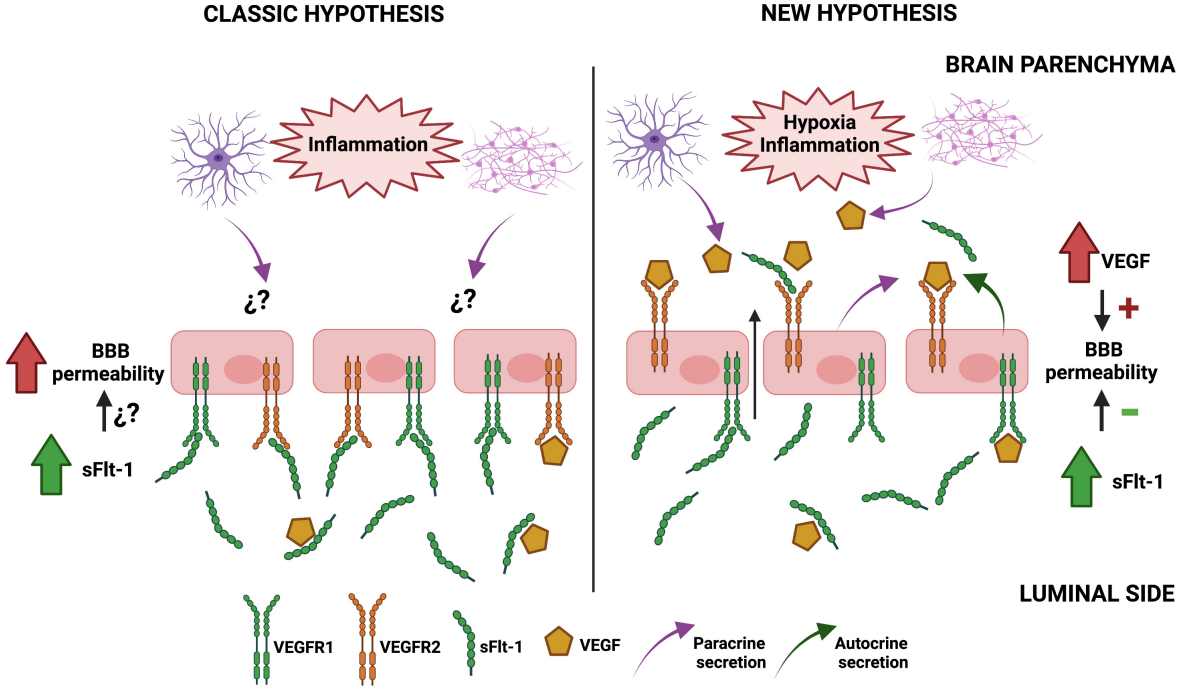

FIGURE 2 | Proposed mechanism of soluble tyrosine kinase-1 similar to fms (sFlt-1) effects on blood-brain barrier (BBB) integrity in preeclampsia. The classic model of endothelial dysfunction in preeclampsia (left) assumes that the alterations on BBB functionality mainly depend on high circulating levels of anti-angiogenic factors such as sFlt-1, as the expression of VEGFRs is similar to the observed in peripheral endothelial cells. However, our hypothesis (right) proposes that the basolateral expression of VEGFR2 in brain endothelial cells makes them more reactive to the paracrine secretion of VEGF from astrocytes and neurons under pro-inflammatory conditions, increasing BBB permeability. Therefore, the high circulating levels of sFlt-1 in the blood of preeclamptic women and the autocrine secretion of this protein in brain endothelial cells might exert a protective effect. The figure omitted the inclusion of other relevant factors in order to better compare both hypotheses. Created with BioRender.com.

pro-inflammatory cytokines including interleukin-1β activate pathways involved in the release of VEGF (Nagineni et al., 2012). In this regard, as ischemic and haemorrhagic stroke are cerebrovascular complications of preeclampsia (McDermott et al., 2018; Too et al., 2018), the paracrine and autocrine activation of VEGFR2 could be responsible of the increased BBB permeability. Indeed, studies have demonstrated that the genomic silencing of VEGF expression in astrocytes abrogates the breakdown of the BBB (Argaw et al., 2012). Therefore, the effects of sFlt-1 on VEGFR2 activity would be in theory protective and evident if the former crosses the BBB or if it is secreted in an autocrine manner (Ahmad et al., 2011). VEGFR1 is not directly involved in BBB permeability, but apparently plays a cytoprotective role in brain endothelial cells (Hudson et al., 2014).

In summary, we propose a challenging hypothesis in which the compartmentalization of VEGF signaling in brain endothelial cells makes them more reactive to the paracrine secretion of this growth factor from brain cells in preeclampsia. As a result, the permeability of the BBB is increased and the high circulating levels of sFlt-1 observed in preeclamptic women may have a protective effect, which is not observed in peripheral endothelial cells such as HUVECs (Zhai et al., 2020). The **Figure 2** presents a graphic description of this hypothesis.

This hypothesis, although plausible, is not without limitations. In our opinion, the lack of strong of evidence regarding the compartmentalization of VEGF signaling in peripheral endothelial cells would difficult the interpretation of findings. Further studies should focus their efforts in conducting comparative studies aiming to characterize the polarization of VEGFRs expression in human brain and peripheral endothelial cells. In addition, the multifactorial nature of preeclampsia suggests that the actions of sFlt-1 on the integrity of endothelial cell monolayers should be assessed under pro-inflammatory, hypoxic and anti-angiogenic environments.

Regarding the influence of inflammation on the pathophysiology of cerebrovascular complications of preeclampsia, neuroinflammation has gained relevance as an outcome that may help to explain certain long-term complications in women who had a preeclamptic pregnancy (Cipolla et al., 2012; Johnson et al., 2014; Clayton et al., 2018). The use of hypoxic conditions would prove useful to analyze the influence of HIF-1α on the reactivity of brain endothelial cells to the effects of sFlt-1. Lastly, the contribution of sEng and the TGF-β pathway to the anti-angiogenic component in preeclampsia's pathophysiology is acknowledged, but despite this body of evidence, the effects at level of the brain vasculature require further characterization.

The above-described studies could be benefited using bi-cameral culturing systems such as Transwell© inserts, which allow co-culturing with other cell types including astrocytes and neurons. Furthermore, the use of human primary cultures or human-derived brain endothelial cell lines would provide more relevant findings or validate previous results collected from animal models. This knowledge will provide a deeper insight on the reactivity of the BBB to pro- and anti-angiogenic conditions. As most of the presented evidence does not always associate the effects of sFlt-1 on the dynamics of VEGFRs function and its downstream events, this gap in knowledge represents an opportunity to better characterize its actions on pathologies including preeclampsia.

CONCLUDING REMARKS

In the last two decades, significant advances have been achieved on the study of the cerebrovascular complications of preeclampsia. There is a better understanding of its pathophysiology, and more recently, this knowledge is being applied to characterize the long-lasting effects of this syndrome on cognition. However, there are many questions to be answered regarding the communication between the placenta and the maternal brain, and the reactivity of brain endothelial cells to noxious circulating factors in preeclamptic women.

The influence of sFlt-1 on VEGF signaling and the development of vascular dysfunction in preeclampsia has not been fully explored at a molecular level, despite its use as a routinary diagnostic biomarker in many countries including the United Kingdom. There is a need for more studies attempting to understand how sFlt-1 alters the dynamics of VEGFRs activation and the crosstalk with other pathways involved in angiogenesis and inflammation. Since most of the current knowledge has been generated from the use of non-cerebral endothelial cell models, we encourage further research in the brain vasculature. If the increase on sFlt-1 levels may eventually be protective instead of harmful factor for the BBB, this knowledge will not only bring more understanding of the pathophysiology of brain complications in preeclampsia, but would help to discovery new therapeutic targets. In particular, the inhibition of VEGFR2 can revert the BBB disruption induced with plasma of women with preeclampsia, but potential therapeutic applicability of these findings has not been even proposed in the literature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

PT-V conceived and wrote the manuscript. RR contributed to the writing of the manuscript. CE contributed to the writing of the manuscript and provided a critical revision of the contents. All authors contributed to the article and approved the submitted version.

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REVIEW
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Programming of Vascular Dysfunction by Maternal Stress: Immune System Implications

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A growing body of evidence highlights that several insults during pregnancy impact the vascular function and immune response of the male and female offspring. Overactivation of the immune system negatively influences cardiovascular function and contributes to cardiovascular disease. In this review, we propose that modulation of the immune system is a potential link between prenatal stress and offspring vascular dysfunction. Glucocorticoids are key mediators of stress and modulate the inflammatory response. The potential mechanisms whereby prenatal stress negatively impacts vascular function in the offspring, including poor hypothalamic–pituitary–adrenal axis regulation of inflammatory response, activation of Th17 cells, renin–angiotensin–aldosterone system hyperactivation, reactive oxygen species imbalance, generation of neoantigens and TLR4 activation, are discussed. Alterations in the immune system by maternal stress during pregnancy have broad relevance for vascular dysfunction and immune-mediated diseases, such as cardiovascular disease.

Keywords: maternal stress, vascular dysfunction, immune response, DOHaD (developmental origins of health and disease), fetal programming, renin–angiotensin–aldosterone system, reactive oxygen species, toll like receptor

Abbreviations: ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AP-1, activator protein-1; AT1, Angiotensin II receptor type 1; Ang-(1–7), angiotensin (1–7); BH4, tetrahydrobiopterin; CRP, C-reactive protein; CXC, chemokines; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL2, chemokine (C-X-C motif) ligand 2; DAMPs, damage-associated molecular patterns; DOHaD, developmental origins of health and diseases paradigm; ET-1, endothelin-1; ET(A), endothelin-1 receptor type A; ET(B), endothelin-2 receptor type B; eNOS, endothelial nitric oxide synthase; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; HIF-1, hypoxia-inducible factor 1; HPA, hypothalamic–pituitary–adrenal axis; ICAM-1, intercellular adhesion molecule-1; IUGR, intrauterine growth restriction; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; IL-15, interleukin-15; IL-17A, interleukin-17A; IRF, type I interferon (IFN)-regulatory factor; lncRNAs, long non-coding RNAs; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; miRNAs, microRNAs; MKP-1, MAPK phosphatase-1 or dual specificity phosphatase-1 (DUSP-1); NADPH oxidase, nicotinamide adenine dinucleotide phosphate hydrogen oxidase; NK, natural killer cells; NF-κB, nuclear factor kappa B; NO, nitric oxide; $\cdot O_2^-$, superoxide anion; oxLDL, oxidized low-density lipoprotein; oxPL, oxidized phospholipids; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species; Th1, T-helper type 1 cell; Th2, T-helper type 2 cell; Th17, T helper-17 cell; TLR, toll-like receptors; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; 11β-HSD2, 11 beta-hydroxysteroid dehydrogenase type 2 enzyme.

INTRODUCTION

Fetal programming is characterized by environmental events experienced during fetal development, inducing immune and inflammatory consequences to the fetus along its lifespan (Facchi et al., 2020). There is consensus that maternal stress during pregnancy is correlated with poor offspring outcomes, and several mechanisms have been recognized to trigger intrauterine disturbances as well as fetal programming.

Maternal stress refers to the chronic or acute psychic, physical or neurological injuries that a mother experiences during any period of the pregnancy and the inability to adapt of these environmental demands (Chrousos and Gold, 1992). Several evidence has linked maternal stress contribute to the development of cardiometabolic disease in later life of offspring, including diabetes and hypertension (Reynolds et al., 2001; Perrone et al., 2016; Simeoni et al., 2018; Briffa et al., 2020). Considering that the immune response is also susceptible to *in utero* programming and that its activation directly contributes to the development of cardiovascular diseases, it is reasonable to hypothesize that the programmed *in utero* immune response may link maternal prenatal stress to vascular dysfunction in the offspring. We propose to explore original manuscripts and conceptual review with potential mechanisms that underpin maternal stress potentially modulates and programs the immune system, inducing vascular dysfunction and cardiovascular diseases in the offspring.

MATERNAL STRESS EXPOSURE: CONTRIBUTION OF GLUCOCORTICOIDS AND INFLAMMATION

During pregnancy, the maternal immune system undergoes an extensive remodeling process, allowing the fetus to adequately grow. Peter Medawar et al. demonstrated the connections between the immune system and organ transplantation, and the immunological paradox of pregnancy was uncovered: "*how does the maternal body sustain a semiallogenic fetus during pregnancy, when it would reject a semi-allogeneic graft?*". Over time, several observations raised the concept that one of the most remarkable features from the maternal immune system is its tolerance capacity, where the mother allows the placenta-fetus unit to develop, avoiding this semi-allograft fetus rejection (Guleria and Sayegh, 2007).

Three different mechanisms were suggested to explain how maternal tolerance occurs during pregnancy (Pang et al., 2016). First, the occurrence of anatomical separation, which was ruled out since a collaborative interaction occurs between maternal decidual leukocytes and the extravillous cytotrophoblast cells from the fetus, promoting the uterine spiral arteries remodeling at early stages of pregnancy (Tilburgs et al., 2015). Second, the possibility of fetal antigens being immature, which was revoked since data from animal studies confirmed that fetal antigens are in fact mature (Billingham et al., 1954). Third, the possibility that the maternal immune system develops a reduced response, a hypothesis that was unsupported since the maternal immune system during pregnancy can respond in a highly effective manner to different pathogens. Currently, the most accepted concept is that trophoblast cells and the maternal immune system develop a cooperative network, favoring the fetal development. Over the course of the pregnancy, distinct immunological phases are observed. In fact, Mor (2008) has concluded that a successful gestation relies on how well the trophoblast will communicate with the maternal immune cells, making them work in synergy (Mor, 2008).

The first trimester of pregnancy is characterized by a remarkable pro-inflammatory environment where the maternal decidua is composed mainly by natural killer (NK) cells and macrophages, with a reduced number of dendritic cells (Bulmer et al., 1988). During this period, uterine innate immune cells display a peculiar phenotype. NK cells are not cytotoxic and are crucial for the regulation of angiogenesis and trophoblast invasion (Kopcow et al., 2005; Hanna et al., 2006). Macrophages favor trophoblast migration and the removal of apoptotic subproducts (Mor et al., 2006). In the second trimester, an anti-inflammatory profile is predominant, with a massive fetal growth and development. In the third immunological phase of pregnancy, the environment is predominantly pro-inflammatory, favoring the parturition process. The immune response readaptation during pregnancy works to prevent the semi-allogeneic fetus rejection, and participates to processes including implantation, placentation, and parturition. Different types of injuries and stress during this phase can harm the development of the fetus and cause great damage in its adult life, programming the development of several disorders and diseases.

In the developmental origins of health and diseases (DOHaD) paradigm, seminal environmental factors are known as endocrine and cardiovascular disruptors that influence the early programming of an unhealthy phenotype. Modulation in gene transcription patterns during critical stages of development such as the intrauterine or suckling phases, which are influenced by maternal exposure to stressful factors, is involved in the origin of many diseases, including obesity, type 2 diabetes, hypertension, asthma, and psychological disorders, that appear in adulthood (Burdge et al., 2007; Heijmans et al., 2008; DeVries and Vercelli, 2016; Agarwal et al., 2018; Tiffon, 2018; Wang et al., 2020).

The food restriction during pregnancy is the most common maternal stress (Smart and Dobbing, 1971; Hales and Barker, 1992; Roseboom et al., 2001). However, obesity, diabetes (Armitage et al., 2008; Shankar et al., 2008; Agarwal et al., 2018), stressful life events, air pollution (Burris and Baccarelli, 2017; Almeida et al., 2019; Wang et al., 2020), nicotine/tobacco smoke (Butler and Goldstein, 1973; Khorram et al., 2010; Santos-Silva et al., 2013; Younes-Rapozo et al., 2013; Lisboa et al., 2017; Peixoto et al., 2018), and the use of synthetic glucocorticoids (Guo et al., 2020; Li et al., 2020; Arias et al., 2021) are important factors influencing the offspring health in later life, since they disrupt maternal and offspring physiology. Although stress induces different signaling pathways, the glucocorticoids are key mediators of stress response (Molnar et al., 2003;

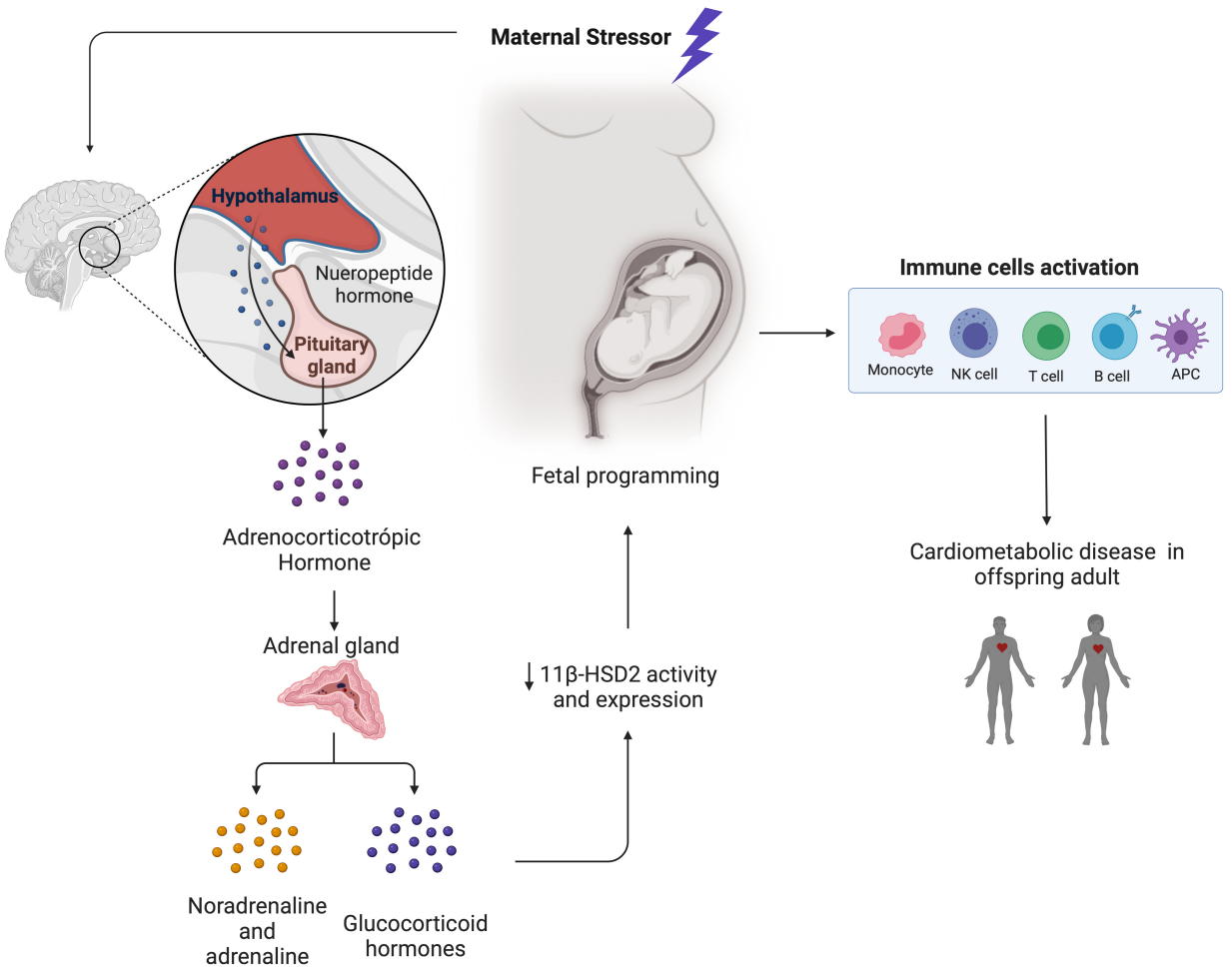

FIGURE 1 | Feedback between maternal stress-induced glucocorticoid production by HPA axis (hypothalamic–pituitary–adrenal axis) activation and fetal programming induced cardiometabolic disease in offspring adult.

Moisiadis and Matthews, 2014a; Facchi et al., 2020) and beside their important role in the acute stress condition, glucocorticoids can also chronically affect the offspring brain neuronal connectivity, stability, and maturation, which can modulate hypothalamic energy controlling pathways (Arck et al., 2001; Sandman et al., 2011; Christian et al., 2013; Solano et al., 2016; Osborne et al., 2018; Schepanski et al., 2018; Nazzari et al., 2020; **Figure 1**).

Glucocorticoid early overexposure: a stress-induced programming-mediating factor. The role of glucocorticoids in mediating early programming of later diseases has been detailed in previous reviews (Lesage et al., 2006; Reynolds, 2013; Moisiadis and Matthews, 2014a,b; Facchi et al., 2020). Experimental and clinical studies support the hypothesis that high maternal cortisol/corticosterone levels as a consequence of stress, as well as decreased placental expression/activity of the fetal protective enzyme 11 beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2), are implicated in the fetal programming of cardiometabolic diseases (Murphy et al., 1974; Bernal et al., 2017). 11β-HSD2 leads to the development of a placental barrier by oxidizing bioactive cortisol/corticosterone into bio-inactive cortisone/11-dehydrocorticosterone and thus avoiding the fetal exposure to elevated cortisol/corticosterone levels.

Maternal food restriction reduces the placental 11β-HSD2 gene expression, imprinting a smaller birthweight and reduced activity of the hypothalamic–pituitary–adrenal (HPA) axis in newborns (Lesage et al., 2001). In addition, reduction in placental 11β-HSD2 gene expression in mothers fed a low-protein diet throughout gestation is associated with smaller birthweight and hyperactive HPA axis, leading to hypertension in weaned rat offspring (Langley-Evans et al., 1996; Langley-Evans, 1997; de Oliveira et al., 2016). Pregnant women under stress conditions display lower placental 11β-HSD2 activity and lower birthweight offspring children (Stewart et al., 1995). Interesting, low activity (Stewart et al., 1995) or deleterious mutation of 11β-HSD2 (Dave-Sharma et al., 1998) was associate smaller weights in the prole. In view of this important mechanism, the deleterious effects of high concentration of glucocorticoid in immune and cardiovascular systems in the prole will be discussed in other sections.

Epigenetic Alterations Induced by Maternal Stress

Epigenetic mechanisms, such as miRNA expression, DNA methylation, and histone modifications are prone to changes in response to stressful experiences and hostile environmental factors (Weaver et al., 2004). Altered epigenetic regulation may potentially influence fetal cardiovascular and immune response programming development across several generations (Ordovas and Smith, 2010; Babenko et al., 2012). Moreover, maternal stress during pregnancy may critically influence the density of glucocorticoid receptors in areas of the fetal brain, particularly the hippocampus, and permanently alter the sensitivity to stress throughout life (Weaver et al., 2004).

Maternal behavior produces stable alterations of DNA methylation and chromatin structure, providing a mechanism for the long-term effects of maternal care on gene expression in the offspring (Weaver et al., 2004). Epigenetic modifications were proposed as probable mechanisms of cardiovascular and metabolic programming (Babenko et al., 2012).

Epigenetic modifications occur after environmental stimuli and play a fundamental role in inflammatory gene transcription (Bayarsaihan, 2011). Indeed, integrative epigenome-wide association studies using large-scale bioinformatics analysis have reported different epigenetic marks related to several circulatory inflammation markers (Gonzalez-Jaramillo et al., 2019). Therefore, epigenetic signature alterations may exacerbate inflammatory responses and influence the risk of chronic inflammatory disease, including cardiovascular diseases (Ramos-Lopez et al., 2021).

Persistent epigenetic changes induced during early exposure to stress conditions may explain the adverse phenotypes expressed later in life (Gluckman et al., 2008). Experimental models of maternal diet during pregnancy presented DNA methylation of specific genes, which resulted in permanent phenotypic changes, such as body weight and blood pressure (Waterland and Jirtle, 2003). During the Dutch Hunger Winter between 1944 and 1945, who individuals were exposed to famine *in utero* had very different methylation patterns in genes involved in growth and metabolic disease compared with controls (Heijmans et al., 2008). These results, combined with evidence of sex-specific methylation patterns suggest that early prenatal undernutrition exposure results in change in DNA methylation and it is an important mechanism of programmed immune and cardiovascular dysregulation.

Early Overexposure to Immune Components: A Stress-Induced Programming Event

Different prenatal stressors increase placental expression of genes involved in the immune response, including interleukin-6 (IL-6) and IL-1β, resulting in male-specific locomotor hyperactivity and increased HPA axis responses (Mueller and Bale, 2008; Bronson and Bale, 2014). A high IL-1:IL-10 ratio, which is implicated in poor coordination of glucocorticoid-immune response, has been observed in the last third trimester of pregnancy (Corwin et al., 2013). As elegantly reviewed by Schepanski et al. (2018), pregnancy stress conditions and infections expose the fetus to neurodevelopmental interferences by many cytokines, increasing the risk for neurological dysfunctions and the early programming for different health disorders in later life (Billingham et al., 1954).

Prenatal maternal stress promove alterations of cytokine profile in offspring at birth (Andersson et al., 2016). Specifically, cytokine profile of umbilical cord blood samples colected at birth from stressed mothers was associated with IL-1β, IL-6, and IL-8, cytokines that all are involved in the pro-inflammatory innate immune response, as well as IL-5 and IL4 that are prototypic cytokines of Th2 response (Wright et al., 2010; Andersson et al., 2016). It is not clear if the early exposure to cytokines high levels can influence the immune system development, but it can have some effect in the prole.

Interleukin-6, derived from the mother after pregnancy stress, effects on fetal microglia by GABAergic progenitor migration and GABAergic system development (Lussier and Stevens, 2016; Gumusoglu et al., 2017). In animal model the offspring from rats that were protein-restricted in diet during pregnancy exhibit pancreatic β-cells with increased apoptotic rate and high susceptibility to the cytokine IL-1β (Merezak et al., 2001).

Glucocorticoids are pivotal to induce physiological changes by influencing gene transcription in many tissues to maintain homeostasis, and by downregulating inflammatory processes through the cytokine-glucocorticoid negative feedback (Newton et al., 2017). By suppressing toll-like receptors (TLR) intracellular signaling, glucocorticoids inhibit the inflammatory process by different mechanisms. Glucocorticoids increase the levels of endogenous inhibitors of TLRs, such as the mitogen-activated protein kinase (MAPK) phosphatase-1 [MKP-1, also known as dual specificity phosphatase-1 (DUSP-1)] and glucocorticoid-induced leucine zipper (GILZ) and inhibit transcription factors that stimulate proinflammatory mediators, such as activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), and type I interferon (IFN)-regulatory factor (IRF) (Chinenov and Rogatsky, 2007).

On the Other hand, some authors have described that the glucocorticoids induce the expression TLR4 TLR2, whose expression is commonly increased by proinflammatory cytokines such as TNF-α and IL-1β (Shuto et al., 2002; Homma et al., 2004; Chinenov and Rogatsky, 2007). In this context, increased TLR2 on epithelial cells stimulates the secretion of the cytokines such as IL-6 and IL-8. A connection between high levels of IL-6 and cardiovascular disease (Qu et al., 2014), as well as an association between IL-8 and high risk of coronary artery disease (Zhang et al., 2019a,b) have also been reported.

Despite the paradoxical crosstalk, it is important to mention that the cellular mechanism by which glucocorticoids downregulate chemokine expression and inflammatory cytokine production, may happen in cases, for example, where NF-κB addresses the systemic inflammation-associated acquired glucocorticoid resistance (Meduri et al., 2005, 2009; Newton et al., 2017). Considering the concept of DOHaD, the lack of glucocorticoids repressor effects on the inflammatory process during stress adversity in critical developmental stages of life may be considered a drawback, since it can contribute to a

proinflammatory environment and negatively influence the relationship between maternal stress during pregnancy and offspring outcomes, as long-term consequences (Elenkov et al., 2005; Newton et al., 2017).

MATERNAL STRESS AND FETAL PROGRAMMING OF CARDIOVASCULAR DYSFUNCTION

Cardiovascular disease is the leading cause of death worldwide (Virani et al., 2021). The vascular system is frequently involved in the pathophysiological processes of cardiovascular diseases. Endothelial dysfunction, inflammation, and remodeling are hallmarks of vascular dysfunction in cardiometabolic diseases, such as diabetes, hypertension, dyslipidemia, and obesity (Petrie et al., 2018). Therefore, the impact, as well as the mechanisms mediating fetal programming in the vasculature have been systematically evaluated.

Vascular dysfunction is characterized by reduction of the capacity of the vessel to maintain the tonus and blood flow, increasing vascular resistance as a result of vascular hypercontractility, reduced vasodilatation, endothelial damage, and vascular remodeling. Several mechanisms may induce vascular dysfunction, including ROS, immune activation, renin–angiotensin–aldosterone system (RAAS), and the sympathetic nervous system (Touyz et al., 2018). The potential mechanisms by which maternal-stress programs vascular dysfunction in adult offspring, contributing to greater incidence of hypertension, will now be discussed.

Food Restriction-Induced Cardiovascular Programming

Food restriction favors low birth weight in humans, correlating with high incidence of cardiovascular disease in adulthood, a feature referred to as the Barker hypothesis or the fetal origins of adult-onset disease (Hoy et al., 1999; Petrie et al., 2018).

The male offspring from pregnant rats submitted to a protocol of diet restriction during pregnancy (50% normal intake) were evaluated from the age of 4 to 14 weeks. Intrauterine food restriction favored high blood pressure levels and a reduced endothelium-dependent relaxation response, and oxidative stress was a contributor to this process (Franco Mdo et al., 2002). Nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase was identified as an important source of superoxide anion ($\cdot O_2^-$) generation, a process that was prevented by *in vitro* NADPH oxidase inhibition (Franco Mdo et al., 2003a), as well as *in vivo* antioxidant treatment of the offspring with ascorbic acid (vitamin C) or alpha-tocopherol (vitamin E) (Franco Mdo et al., 2003b). Prenatal antioxidant vitamin supplementation in rats exposed to a food restriction protocol during pregnancy prevented blood pressure increases and restored endothelium-dependent relaxation in the adult offspring (Franco Mdo et al., 2009). *In vitro* infusion with tetrahydrobiopterin (BH4), a cofactor for endothelial nitric oxide synthase (eNOS) activity, reverted endothelial dysfunction, favoring nitric oxide (NO) production and reduction of oxidative stress in the microvasculature (Franco Mdo et al., 2004).

Interestingly, reduced leukocyte migration was observed in offspring (8–9 weeks of age) from undernourished mothers, as a result of reduced adhesion molecule expression in leukocytes (L-selectin) and endothelial cells [P-selectin and intercellular adhesion molecule-1 (ICAM-1)] (Landgraf et al., 2005). Vascular structure may also be affected by intrauterine undernutrition, which leads to vascular smooth muscle cell hypertrophy in adult offspring (Khorram et al., 2007).

Sex-specific differences were observed in 6-month old adult offspring from mothers submitted to an undernutrition protocol during pregnancy. While males displayed augmented blood pressure levels and increased plasma metalloproteinase-9 activity, in females no differences were observed. However, females displayed smaller mesenteric resistance artery diameter, reduced cellularity of smooth muscle cells, among other structural alterations, showing that undernutrition favors later life microvasculature remodeling in both sexes, regardless of changes in blood pressure levels (Gutierrez-Arzapalo et al., 2020).

Intrauterine growth restriction (IUGR) imposed to baboons *(Papio species)*, using a protocol of 70% of food restriction during pregnancy to the end of lactation showed alterations in vascular parameters of young adult offspring, without changes in blood pressure. Augmented systolic velocity, reduced femoral and iliac artery lumen area, and reduced external iliac artery distensibility were observed in male IUGR offspring. These observations suggest that vascular remodeling observed in IUGR during fetal life somehow modulates vascular alterations later in life (Kuo et al., 2018).

Obesity-Induced Cardiovascular Programming

Epidemiological studies demonstrated that maternal nutrient restriction or overnutrition during pregnancy predispose offspring to a much higher prevalence of obesity, glucose intolerance, insulin resistance, hypertension, vascular dysfunction, and heart disease (Rankin et al., 2010; Dong et al., 2013; Zambrano and Nathanielsz, 2013). While maternal nutrient restriction during pregnancy may trigger an increase in the tissue-specific glucocorticoid receptor, the maternal obesity induced increase in fetal hormones such as leptin and insulin, nutrients such as fatty acids, triglycerides and glucose, and inflammatory cytokines (Whorwood et al., 2001; Sullivan et al., 2011). Prenatal dietary fat exposure predisposes offspring to postnatal dietary fat-induced cardiac hypertrophy and contractile defects possibly *via* lipotoxicity, glucose intolerance, and mitochondrial dysfunction (Turdi et al., 2013). Clinical studies observed that the obesity during pregnancy induced elevation of blood pressure and cardiovascular disease in adult offspring (Gaillard, 2015).

Moreover, the maternal nutritional environment is closely associated with fetal heart and vascular development and function (Dong et al., 2008). Maternal obesity impairs fetal cardiomyocyte contractility through altered intracellular Ca^{2+} handling, overloading fetal cardiomyocyte intracellular Ca^{2+} and aberrant myofilament protein composition in sheep (Wang et al., 2019). Rats exposed *in utero* to mild maternal diet-induced obesity presented specific epigenetic modulations of matrix

metalloproteinases, collagens, and potassium channels genes in association with an outward remodeling and perturbations in the endothelium-dependent vasodilation pathways in small mesenteric arteries (Payen et al., 2021).

Hypoxia-Induced Vascular Programming

Fetal hypoxia has been linked to fetal programming of cardiovascular, renal, cerebral, and metabolic dysfunctions. Intrauterine hypoxia is frequently related to IUGR and can be induced for different maternal stress, including food restriction or obesity. It is reasonable to link oxidative stress and impairment of vascular development as mechanisms mediating intrauterine hypoxia, considering that placental dysfunction creates an ischemic environment (Fajersztajn and Veras, 2017).

Adult offspring coming from a hypoxic-environment during pregnancy associate to postnatal salt dietic display reduced endothelium-dependent relaxation. Still, larger vessels in these animals exhibit remodeling characterized by augmented collagen deposition, and reduced elastin (Walton et al., 2016). Similar observations were made in rat aortas, along with cardiac remodeling (Benny et al., 2020). IUGR also favors arterial remodeling in the adult offspring, as observed in sheep (Dodson et al., 2014).

Gestational intermittent hypoxia leads to vascular dysfunction in male offspring, due to reduced anti-contractile activity of perivascular adipose tissue, *via* epigenetic modification on the adiponectin gene promoter (Badran et al., 2019). Low oxygen levels activate the hypoxia-inducible factor 1 (HIF-1) complex, inducing epigenetic regulation, a topic that will be addressed later in this review.

Glucocorticoid-Induced Vascular Prenatal Programming

Glucocorticoid production is important during pregnancy. In normal pregnancies, cortisol raises significantly close to the pregnancy midterm, reaching levels around 350 ng/mL (Wieczorek et al., 2019) and remaining relatively stable until labor, when cortisol raises again (Carr et al., 1981). The abundance of glucocorticoids occurs to ensure fetal organs maturation close to birth (Solano et al., 2016). Its excess is buffered in the maternal blood by the corticosteroid-binding globulin transporters, globulins that raise during pregnancy (Kumsta et al., 2007). In fact, stress-induced secretion of glucocorticoids impacts on energy stores mobilization, enhances neural function and impact on cardiovascular responses (Sapolsky et al., 2000). Yet, glucocorticoid receptors are expressed in the uterus and the target deletion of these receptors in the uterus results in infertility, inflammation and poor decidualization due to incorrect immune cell recruitment (Whirledge et al., 2015).

High doses of exogenous glucocorticoids have been used in experimental models to directly assess the role of glucocorticoids in triggering prenatal stress conditions. Therefore, intrauterine glucocorticoid exposure due to maternal stress conditions and exogenous glucocorticoid sources causes deleterious effects on vascular function in later life (Lamothe et al., 2021) and alterations of vasoactive substances may be involved.

Early gestational dexamethasone administration augments offspring blood pressure levels in sheep and favors increased contractile response to angiotensin II (Ang II), phenylephrine and thromboxane analogues (U-46619) in coronary arteries (Roghair et al., 2005). Augmented contractile response to Ang II was partially mediated by endothelial $\cdot O_2^-$ overproduction, through enhanced NADPH oxidase activity (Roghair et al., 2008). The mechanisms contributing to hypertension in a similar experimental model were related to compensatory endothelial-dependent vasodilation, augmented sympathetic activity, and baroreflex adaptation (Segar et al., 2006). Importantly, fetal programming of hypertension involves hyper-sympathetic activation of the Ang II type 1 (AT1) receptor activated by endogenous Ang II (Vieira-Rocha et al., 2019). These and other observations support the concept that activation of the renin–angiotensin system plays a role in vascular dysfunction in the adult offspring exposed to glucocorticoids during fetal life (Hsu and Tain, 2021).

Not only intra-uterine, but also early life stress may result in vascular programming, affecting the RAAS system. Adult offspring from mothers submitted to litter separation are more susceptible to hypertension in response to chronic administration of Ang II, as well as to vascular inflammation (Loria et al., 2010) and vascular constriction to Ang II (Loria et al., 2011). Conversely, some protective effects may also be initiated by maternal separation. For example, the capacity of the perivascular adipose tissue to modulate vasoconstriction and endothelial dysfunction increased (Loria et al., 2018). Yet, brief periods of daily maternal separation lead to impaired resistance artery due augmented vascular contractility-response and higher blood pressure in adult rats (Reho and Fisher, 2015). Even the pulmonary vasculature may be affected by stressor conditions, including cross-fostering in early natal period, through renin–angiotensin system modulation (Shifrin et al., 2015).

Renin–angiotensin–aldosterone system may also be a target of fetal programming. The imbalance between the ratio of plasma Ang II to Angiotensin 1–7 [Ang-(1–7)], along with a gradual reduction of Ang-(1–7) were observed in adult offspring from sheep treated with betamethasone during pregnancy, and these alterations occurred prior to blood pressure increases (Shaltout et al., 2012). Yet, antenatal exposure to betamethasone caused a persistent reduction in serum and kidney angiotensin-converting enzyme (ACE)-2 activity, favoring higher blood pressure levels and decreased Ang-(1–7) levels (Shaltout et al., 2009). The impact of fetal programming on the Ang-(1–7) axis was recently reviewed (South et al., 2019).

Dexamethasone administration in fetal life resulted in a compensatory response of endothelium-dependent relaxation, as well as increased contractile response to endothelin-1 (ET-1) (Molnar et al., 2003). Adult offspring from betamethasone-exposed mothers displayed augmented blood pressure, and ET-1 increased blood pressure and vascular resistance *via* ET(A) receptor (Lee et al., 2013). Yet, impaired ET(B) receptor activity favored augmented ET-1 contractility in vascular smooth muscle

cells, along with a decrease in ET-1-induced endothelium-dependent relaxation (Pulgar and Figueroa, 2006).

Epigenetic Events and Vascular Prenatal Programming

Epigenetic events connect the intrinsic genetic background with the extrinsic environment, resulting in the attachment of chemical groups in the DNA, modulating gene expression. The most frequent and described DNA modifications include DNA methylation or hydroxymethylation, histone modifications, and chromatin packaging in addition to the occurrence of microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) (Rane et al., 2009; Deng et al., 2015; Shi et al., 2015).

A growing body of evidence supports the idea that glucocorticoid-related genes involved in augmented cardiovascular risk factors in adult life are targeted by DNA methylation, resulting from early life stressful environment (Friso et al., 2008; Reynolds et al., 2013; Tobi et al., 2018). Another major issue is how the placenta may be affected by epigenetic modifications. In this regard, microRNA interference, DNA methylation, as well as histone methylation suppress placental 11β-HSD2 and GR genes, favoring augmented glucocorticoid levels (Togher et al., 2014).

Considering that undernutrition imposes IUGR and a hypoxic environment to the fetus, the absence of oxygen may regulate a variety of oxygen-regulated genes, *via* HIFs (Adams et al., 2009). miRNAs related to renin–angiotensin system components are affected by maternal hypoxia, and this genetic reprogramming may be involved in the development of pulmonary hypertension in adult offspring (Goyal et al., 2011).

Environmental factors, including ethanol exposure during intrauterine life also result in cardiovascular reprogramming. Ethanol exposure in uterine life reduces 11β-HSD2 expression in the rat placenta and exposes the fetus to glucocorticoids overproduction, by a mechanism that increases permeability of the placental barrier (Zhang et al., 2014). Offspring displayed elevated blood pressure in adulthood and endothelium-dependent and -independent vasodilation was impaired in rats (Turcotte et al., 2002). Morphologically, the vasculature was also modified, and offspring from mothers exposed to ethanol during pregnancy displayed augmented arterial stiffness (Parkington et al., 2014).

Epigenetic alterations, such as DNA methylation and histone modification may be observed upon ethanol consumption (Kleiber et al., 2014; Chater-Diehl et al., 2017). In this regard, long-term DNA methylation was investigated in children displaying neurological and multiple tissue injuries related to prenatal ethanol exposure, and a singular profile of DNA methylation was observed (Laufer et al., 2015). Experiments using the Agouti viable yellow mouse model showed that intrauterine exposure to ethanol generated DNA methylation in the adolescent offspring, as indicated by coat color change (Kaminen-Ahola et al., 2010).

Smoking during pregnancy represents another major concern of how environmental exposure may affect vascular programming. Although most of the data supporting deleterious effects of smoking comes from cigarette smoking, electronic cigarettes may also be important players on fetal programming (Holbrook, 2016). Exposure to nicotine during intrauterine life resulted in DNA methylation of miR-181 contributing to the cardiac ischemia sensitive phenotype in adult rats, favoring autophagy signaling pathways (Jian et al., 2020). AT1 and AT2 receptors gene expression in adult rat offspring from nicotine-exposed mothers is modulated by DNA methylation, eliciting high blood pressure and augmented vascular contractility in adulthood (Xiao et al., 2014). Impaired vascular reactivity due to antenatal exposure may be due to excessive ROS production, *via* Nox-2 dependent mechanisms (Xiao et al., 2011). Translational data showed that preschool children exposed to tobacco during pregnancy display normal blood pressure, but, surprisingly, their systolic blood pressure is increased (4.2 mmHg), compared to that in children with similar age/height that were not exposed to tobacco (Nordenstam et al., 2019).

Recently, particulate matter was also recognized as a possible environmental player in intrauterine stress and programming. On top of the gestational impact that ultrafine particles exposure generates, it also favors programming *via* DNA hypomethylation, eliciting fetal activation of promoters of RAAS, including AT1 receptor and ACE, contributing to increased blood pressure in adult mice offspring. Placental levels of 11β-HSD2 were reduced, whereas maternal and fetal cortisol levels were increased, in the ultrafine particles-exposed group (Morales-Rubio et al., 2019).

In the future, the use of epigenetic markers may be very useful to select the population who is under high risk for cardiovascular events due to intrauterine maternal stress, using biomarker-guidance interventions as a strategy to closely follow these higher risk patients. Remarkably, placental epigenetic biomarkers may also be used to map populations that might be in high cardiovascular risk in adult life.

MATERNAL STRESS AND FETAL PROGRAMMING OF IMMUNE RESPONSE

Multiple studies suggest that chronic maternal stress plays have deleterious effects on fetal and neonatal immune functions. Repeated maternal stress significantly alters offspring leukocyte function (Coe et al., 2002), affects placental transfer of maternal antibodies in a sex-dependent manner (Coe and Crispen, 2000), and increases susceptibility to infection (Kay et al., 1998). Although some elements have already been described, it is difficult to define the profile of the programmed immune response due to the different experimental models and the different results described. This chapter highlights the main mechanisms by which maternal stress programs the immune response in the offspring.

When discussing changes in the immune response due to programming originated from maternal stress in the prenatal period, it is essential to consider the windows of vulnerability. These are moments during cell development, where there is a continuous change in gene expression and in the profile of molecules that result in specific time frames in which

some cellular elements become more sensitive than others (Veru et al., 2014). Another important observation is that the development and maturation of immune system components occur at different times depending on the studied species. In general, in animals that give birth to preterm offspring (sheep, pigs, guinea pigs, and primates) the development of the immune system occurs predominantly *in utero* (Holladay, 1999; Holladay and Smialowicz, 2000; Merlot et al., 2008). By contrast, in species that give birth to non-preterm offspring (rats, rabbits, and mice), a large proportion of the development occurs during late gestation and in the postnatal period (Holladay, 1999; Holladay and Smialowicz, 2000). For example, the capacity of T cells from 3-day-old monkeys to respond to non-self antigens in mixed lymphocyte cultures is decreased in babies whose mothers were stressed during mid-late pregnancy, and increased in babies whose mothers were exposed to the same stressor during early pregnancy. This reveals that the direction of the alteration depends on the time of exposure to the stressor during pregnancy.

Another factor to consider in the immune response programmed by maternal stress is the type and duration of the insult. A metanalysis of over 300 studies found that different types of stress impact different aspects of immune function. Acute laboratory stressors upregulate innate immunity and downregulate adaptive immunity. Brief exposure to naturalistic stressors, such as academic examinations, shifts function away from cellular immunity (T-helper type 1; Th1) and toward humoral immunity (Th2); and chronic stressors, which are pervasive and insistent, suppress both innate and adaptive immunity (Segerstrom and Miller, 2004).

Epigenetics mechanisms, such as DNA methylation, can modify the immune response across the lifespan in response to maternal stress during pregnancy (Al-Hussainy and Mohammed, 2021). T cell differentiation is controlled by epigenetic mechanisms that regulate Th1 and Th2 identity. Prenatal maternal psychological stress can affect the epigenetic profile of human T cells in a way that affects cytokine production (Al-Hussainy and Mohammed, 2021). Cao-Lei et al. (2014) examined the DNA methylation patterns in T cells of offspring of women exposed to the 1998 Quebec ice storm and found that the genes most commonly methylated are involved in immune system pathways (Cao-Lei et al., 2014). Studies of rhesus macaques revealed differentially methylated regions in both T cells and prefrontal cortex in monkeys who were separated from their mothers after birth (Provencal et al., 2012).

Most studies on immune response dysregulation by maternal stress report increased risk of asthma and allergic diseases in offspring (Douros et al., 2017). A study performed in Northern Italy, in a population of 3,854 children, found that children of mothers who had experienced stressful life events during pregnancy, exhibit moderately increased risk of having wheezing, asthma, eczema and allergic rhinitis during their childhood (de Marco et al., 2012). In fact, maternal prenatal stress can lead to a disequilibrium of Th1/Th2 ratio, in favor of Th2 response, increasing IL-4 and IL-5 in the offspring. and favoring the development of atopic diseases (Pincus-Knackstedt et al., 2006; Veru et al., 2014; Douros et al., 2017).

However, a study performed by Robinson et al. (2021) examined the relationship between the nature and timing of maternal stress in pregnancy and hospitalization due to infections in the offspring. They included 2,141 offspring in the study and the mothers were asked at 18 and 36 weeks gestation about psychological stress events, such as death of a close friend or relative, separation, marital problems, job loss, money problems, and others. The authors found an association between the timing and the type of stress with infection-related hospitalizations. Surprisingly, hospitalizations were observed only in male offspring, showing a sex-specific risk of severe infection in offspring by exposure to maternal prenatal stress. A potential mechanism suggested is that chronic stress may program a pro-inflammatory phenotype in monocytes and macrophages, increasing susceptibility to infections, possibly *via* the hypothalamic–pituitary axis (Marvar et al., 2010).

In addition, study involving 66,203 mother-child pair, emotional stress during pregnancy was associated only with an increased risk of infectious disease (Tegethoff et al., 2011). Elevated stress levels across pregnancy have also been associated with changes in production of pro-inflammatory cytokines in the offspring. Laviola et al. (2004) used a prenatal stress model where rats underwent three 45-min session per day of prenatal restraint stress on gestation days 11–21. Adolescent offspring exhibited higher IL-1β and decreased IL-2 concentrations in spleen and decreased circulating CD4 T cells, CD8 T cells, and CD4/CD8 ratio compared with stress-free animals. Interestingly, an environmental intervention, enriched housing, reversed most immunological alterations, leading to increased IL-2, reduced IL-1β in splenocytes and increased CD4 and CD4/CD8 ratio (Laviola et al., 2004). These findings suggest that the immunological response can be changed by events occurring at multiple stages in development.

Prenatal maternal stress can also impact the neonatal adaptive immunity along generations. Garcia-Flores et al. (2020) used a murine model of prenatal maternal stress across three generations. The authors applied four stress procedures including swimming, restraint, shaking, and white noise for one week in 10 days post-coitum in three generation of dams and evaluated the adaptive immune response of neonates. Results showed a reduction in T cells and B cells, including regulatory CD4 T cells as well as IL-4 and IL-17A producing T cells only in the second generation, but such effects are restored in the third generation. The authors propose that this response is a compensatory mechanism against prenatal maternal stress.

Although this study did not observe an increase in IL-17A in the offspring of stressed pregnant animals, some studies describe the association between IL-17A and chronic stress (Liu et al., 2012; Swardfager et al., 2014; Nadeem et al., 2017). Increased levels of IL-17A measured at 24–28 week gestation were associated with lower birthweight, lower emotional intelligence, lower antenatal maternal attachment, higher prenatal distress, and higher number of life events in pregnant women (Moore et al., 2019).

The dysregulation of the autonomic nervous system is a hallmark of many psychological disorders and elevated levels of norepinephrine directly lead to increased production of IL-17A from T cells (Case et al., 2016). In fact, the central nervous system plays an orchestrated role in inflammation, inducing a direct impact on inflammatory cytokines and immune cells. The central

inhibition of the sympathetic nervous system (SNS) decreased peripheral TNF-α serum levels in hypertensive post-menopausal women (Pöyhönen-Alho et al., 2008). Suppression of the adaptive immune system or sympathetic outflow inhibits hypertension (Rodríguez-Iturbe et al., 2002; Lob et al., 2010), and enhances T cell activation (Marvar et al., 2010) in experimental models. Renal sympathetic nerves play an important role in activation of adaptive immunity in hypertension by T cell activation. In fact, renal denervation not only reduces the total number of immune cells in the kidney, but also memory T cells (Xiao et al., 2015). The immune cells present adrenergic receptors, which have been implicated in sympathetic response in the inflammatory response. Has been reviewed by Kohm and Sanders (2001) that NE and beta 2-adrenergic receptor contributed to CD4 + T and B lymphocyte regulation.

Another mechanism by which the immune response is altered by fetal programming is oxidative stress. ROS generation is an autolimited mechanism of inflammation. However, prolonged inflammation and disturbance in immune homeostasis can lead to an oxidative stress condition and more severe damage of cellular and tissue structures. Restraint stress at late-stage of pregnancy in rats caused increased intracellular ROS, loss of hippocampal neurons and activation of NF-kB signaling (Zhu et al., 2004; Cai et al., 2007). In the same animal model, oxidative damage in mitochondrial DNA in hippocampal neurons only in females rat offspring was reported (Song et al., 2009). Higher levels of glucocorticoid exposure by 11β-HSD inhibitor, during the last week of murine gestation, increased aortic superoxide anion production (Roghair et al., 2011). Treatment of the pregnant mice with Tempol, an antioxidant, did not correct glucocorticoid-programmed aortic superoxide production, but attenuated conditioned fear and stress reactivity.

Maternal obesity can also affect the inflammatory response in the offspring. Obesity, *per se*, is known to be a chronic inflammatory state and C-reactive protein (CRP) is elevated in non-obese adult offspring of two obese parents (Lieb et al., 2009). In another study, levels of high sensitivity-CRP were higher in 12-year-old children exposed to maternal obesity during pregnancy compared to not exposed (Leibowitz et al., 2012). In a study of 189,783 Swedish children, a higher maternal body mass index (BMI) was associated with a higher risk of asthma (Lowe et al., 2011).

Despite the variation in the immune response programmed by maternal stress *in utero*, the most consistently replicated findings have been observed in cytokine homeostasis, where prenatal maternal stress induces a Th2 cytokine shift (adaptive immunity) and excessive pro-inflammatory cytokine responses (innate immunity).

PROGRAMMED IMMUNE RESPONSE CONTRIBUTING TO VASCULAR DYSFUNCTION

Most cases of arterial hypertension have an idiopathic and multifactorial cause (Whelton et al., 2018). The contribution of immune response activation to increased blood pressure and cardiovascular diseases has already been well described and revised (Bomfim et al., 2017). Thus, the focus of this chapter is to connect the mechanisms of the immune response that contribute to vascular dysfunction and that can be programmed by maternal stress during pregnancy. Here, potential mechanisms whereby programmed immune response induced by maternal stress trigger vascular dysfunction and increase the risk of cardiovascular disease in adulthood will be discussed (**Figure 2**).

Poor Hypothalamic–Pituitary–Adrenal Axis Regulation of Inflammatory Response

Glucocorticoids are classically known for their immunosuppressive effect. However, several studies have also shown that chronic treatment with synthetic corticosteroids increases blood pressure (Goodwin and Geller, 2012). The main mechanisms by which corticosteroids increase blood pressure are sodium reabsorption in the proximal tubules of the kidneys, and increased intravascular fluids due to glucocorticoid-induced activation of mineralocorticoid receptors. High doses of dexamethasone reduce 11β-HSD2 expression, increasing susceptibility to hypertension (Bailey, 2017). In addition, glucocorticoid receptors are expressed in vascular smooth muscle cells and endothelium, and their activation changes the vascular behavior, with increased contraction and decreased vascular relaxation. However, little is known about the role of glucocorticoid receptors in these sites (Grossman and Messerli, 2012).

Despite the fact that glucocorticoids have an immunosuppressive function, some studies have shown a pro-inflammatory action of this hormone (Shimba and Ikuta, 2020a,b). One of the mechanisms for the constant activation of the immune system, even at high concentrations of glucocorticoids, is the poor regulation of cytokine-glucocorticoid negative feedback, which influences the relationship between maternal stress during pregnancy and offspring outcomes (Hantsoo et al., 2019). A well-regulated, flexible immune system responds appropriately to glucocorticoid stimulation, and a brief spike in pro-inflammatory cytokines in response to acute stress is considered physiologically appropriate. However, an exaggerated or prolonged immune response is maladaptive, and chronic stress may result in glucocorticoid receptor resistance, the sensitivity of the immune cells to glucocorticoid hormones and inflammatory response (Cohen et al., 2012). Without sufficient glucocorticoid regulation, the duration and/or intensity of the inflammatory response increases. Vascular dysfunction is correlated with elevated serum levels of inflammatory markers and increased immune response (Shao et al., 2020), and glucocorticoid receptor resistance increases the intensity and duration of inflammatory processes, further contributing to vascular dysfunction and heightening risk and progression of cardiovascular disease results in failure to downregulate inflammatory response.

Activation of T Helper-17 Cells

T helper-17 (Th17) cells, a unique CD4+ T-cell subset, are important in the secretion of IL-17, IL-21, and IL-6. Apart

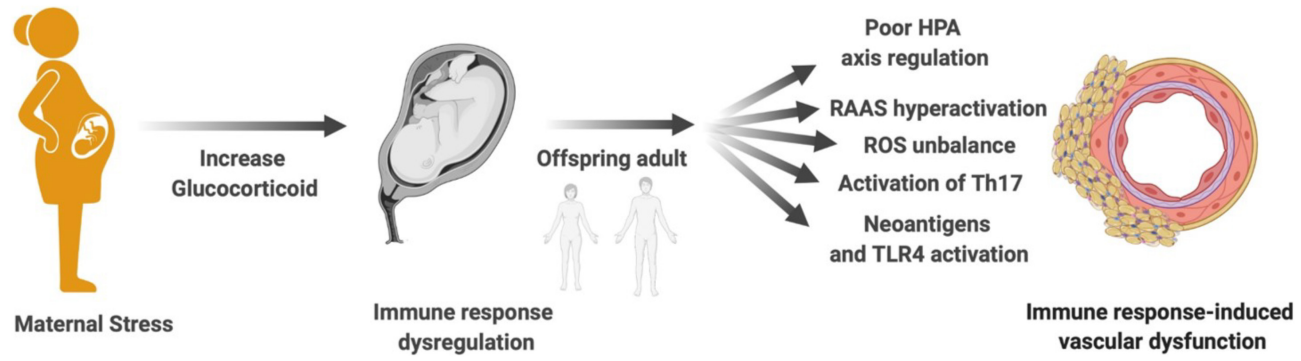

FIGURE 2 | Maternal stress, immune response, and vascular dysfunction. Mechanisms proposed to explain how maternal stress programming-induced immune response dysregulation in the offspring contributes to vascular dysfunction in adult life: poor HPA axis regulation, RAAS hyperactivation, ROS imbalance, activation of Th17, generation of neoantigens, and TLR4 activation. HPA, hypothalamic–pituitary–adrenal; RAAS, renin–angiotensin–aldosterone system; ROS, reactive oxygen species; Th17, T helper lymphocyte 17; TLR4, toll-like receptor 4.

from Th17 cells, selective cell subtypes also produce IL-17, including γδ T cells, NK, and NK-T. IL-17A signaling activates various downstream pathways, which include MAPK and NF-κB to induce mediators with relevance to cardiovascular risk such as CXC chemokines, CXCL1 and CXCL2, involved in the attraction of neutrophils, and inflammatory factors, like IL-6 and granulocyte–macrophage colony-stimulating factor (Tesmer et al., 2008).

In the inflammation-induced stress system, the production of Th1/pro-inflammatory cytokines by Th2 protects the organism from systemic "overshooting" (Elenkov et al., 2005). Although in the Th1/Th2 dichotomy the Th2 response predominates in animals whose mother was submitted to stress, glucocorticoids have also the potential to enhance the development and function of Th17 cells (Shimba and Ikuta, 2020a). Th17 cells are resistant to apoptosis and to the suppression of cytokine production by glucocorticoid treatment (Banuelos et al., 2017). Thus, stress-induced glucocorticoids might trigger inflammation *via* induction of Th17 cells (Shimba and Ikuta, 2020a). Plasma levels of IL-17A are increased in humans with hypertension (Madhur et al., 2010). Mice lacking IL-17A develop blunted hypertension and do not develop endothelial dysfunction in response to Ang II infusion. IL-17A can inhibit endothelial NO production, increase ROS formation, and promote vascular fibrosis contributing to vascular dysfunction (Davis et al., 2021). IL-17A can also cause autoimmunity through an IL-6 positive feedback loop (Ogura et al., 2008). Thus, it is reasonable to hypothesize that Th17 cells are more activated in the offspring of mothers that underwent stressful insults during pregnancy, and that Th17 cells contributes to the onset of vascular dysfunction and cardiovascular disease in adulthood.

Renin–Angiotensin Aldosterone System Activation

The Ang II axis of the RAAS is recognized as a key pathway in the cardiovascular pathology of fetal programming events. Franco Mdo et al. (2003a) showed that Ang II concentration is increased in mesenteric arteriolar beds from intrauterine undernourished rats. The same experimental model exhibits augmented ACE activity (Langley-Evans et al., 1999). Reduced ACE2 activity and increased Ang II/Ang-(1–7) ratio were found in adult sheep offspring whose mothers were exposed to betamethasone, in the third trimester of pregnancy. Therefore, exposure to glucocorticoids, likewise betamethasone, programs a dysfunctional RAAS (South et al., 2019), implicating in higher long-term risk for hypertension and cardiovascular disease.

Angiotensin II acts mainly through AT1 receptor and activates many mechanisms in the kidney, nervous system, and blood vessels that cause hypertension. Besides its classic role in the regulation of circulatory homeostasis, it is very well described that Ang II is also a powerful pro-inflammatory mediator (Biancardi et al., 2017). Ang II inflammatory effects are mediated by activation of NF-κB, and the production of inflammatory mediators, including interleukin IL-1β, IL-6, and TNF-α (Marchesi et al., 2008). Ang II, *via* AT1 receptor, also stimulates NADPH oxidase (Nox enzymes) and generates ROS in the blood vessels (Schiffrin and Touyz, 2003). In response to Ang II, the expression of several inflammatory chemokines and their receptors are increased in the target organs, including the vasculature, mediating homing of immune cells (Mikolajczyk et al., 2021). Therefore, it is plausible to suggest that the RAAS is an important immumodulator that contributes to vascular dysfunction in offspring due to maternal stress in the prenatal period.

Reactive Oxygen Species Imbalance

As already discussed, maternal stress *in utero* increases ROS generation and leads to chronic oxidative stress in various organs of the adult offspring. The production of ROS is strongly stimulated during activated immune responses, due to the important microbicidal activity of ROS. However, the exaggerated and chronic ROS production damages the vascular system. There are excellent reviews covering the role of oxidative stress, vascular dysfunction, and hypertension (Higashi et al., 2014; Montezano et al., 2015; Griendling et al., 2021). In general, oxidative stress favors a vasoconstrictor, mitogenic, pro-fibrotic,

pro-migratory, and pro-inflammatory phenotype in endothelial cells and vascular smooth muscle cells (Montezano et al., 2015). With this in mind, it is very likely that adults will develop vascular dysfunction triggered by programmed oxidative stress during the pregnancy of stressed mothers.

Generation of Neoantigens and Toll-Like Receptor-4 Activation

In addition to directly causing vascular dysfunction, oxidative stress, or the excess of ROS can oxidize endogenous molecules and generate damage-associated molecular patterns (DAMPs), such as oxidized low-density lipoprotein (oxLDL) and oxidized phospholipids (oxPL) (Rocha et al., 2016). oxLDL binds to TLR4, which is a key signaling receptor of innate immunity (Miller et al., 2005). TLR4 signaling leads to activation of MAPK and NF-κB, resulting in secretion of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, and others (Kawai and Akira, 2006). oxLDL activates TLR4 and induces secretion of chemokine IL-8 and monocyte chemoattractant protein-1 (MCP-1) by human endothelial cells and monocytes (Miller et al., 2005). In addition, oxLDL/LDL-C ratio, an atherogenic index, is increased in IUGR fetuses compared to neonates of healthy mothers with appropriate weight.

Activation of TLR4 is directly involved in vascular inflammation, vascular dysfunction, and hypertension (Bomfim et al., 2012, 2015). Vascular TLR4 expression is increased in cardiovascular disease, including several hypertension (SHR, Ang II infusion, DOCA-Salt), atherosclerosis, and other animal models (Roshan et al., 2016; Biancardi et al., 2017). Treatment with a neutralizing anti-TLR4 antibody decreases blood pressure and vascular inflammation in hypertensive rats (Bomfim et al., 2012, 2015). TLR4 overexpression aggravates vascular smooth muscle cells proliferation and vascular remodeling in the pathogenesis of hypertension (Qi et al., 2021).

In view of all these information, we suggest an alternative mechanism involved in vascular dysfunction programmed by immune response in offspring submitted to stress *in utero*: the generation of neoantigens by oxidative stress and activation of TLR4.

CONCLUSION

In conclusion, this review proposes a connection between maternal stress, immune response and vascular dysfunction. During pregnancy, stressors occurring in different gestational periods activate immune responses in the offspring, favoring a poor HPA axis regulation of inflammation, RAAS activation, unbalanced ROS, and generation of new antigens and activation of TLR4. All these mechanisms are directly involved in vascular dysfunction and cardiovascular disease development.

Thus, a careful attention/assistance to women during pregnancy and the nursing period is essential to program a healthy cardiometabolic phenotype in the offspring. In fact, by giving special attention to women living in socially unassisted communities is an urgent necessity around the world. Strategies to implant social policies that can improve the nutritional status, educational strategies to avoid the use of licit drugs like alcohol and tobacco, as well as especial attention to the psychiatric and behavioral complications in this stage can avoid maternal stress and the long-term impact of negative events in the offspring adult life. Avoiding maternal stress is an important step to prevent the onset of cardiovascular diseases in the offspring and their long-term consequences.

AUTHOR CONTRIBUTIONS

GB, TC, FG, and JD conceptualized the idea for the manuscript. GB, TC, FG, JD, and VL performed the literature search and drafted different sections of the manuscript. RT, GB, and TC revised and edited the final version of the manuscript. TC drafted the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Severe Acute Respiratory Syndrome Coronavirus 2 Infection in Pregnancy. A Non-systematic Review of Clinical Presentation, Potential Effects of Physiological Adaptations in Pregnancy, and Placental Vascular Alterations

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In December 2019, the novel Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) rapidly spread to become a pandemic. To date, increasing evidence has described the potential negative impact of SARS-CoV-2 infection on pregnant women. Although the pathophysiology of coronavirus disease 2019 (COVID-19) is not entirely understood, there is emerging evidence that it causes a severe systemic inflammatory response associated with vascular alterations that could be of special interest considering some physiological changes in pregnancy. Additionally, these alterations may affect the physiology of the placenta and are associated with pregnancy complications and abnormal histologic findings. On the other hand, data about the vaccine against SARS-CoV-2 are limited, but the risks of administering COVID-19 vaccines during pregnancy appear to be minimal. This review summarizes the current literature on SARSCoV2 virus infection, the development of COVID-19 and its relationship with physiological changes, and angiotensin-converting enzyme 2 (ACE2) function during pregnancy. We have particularly emphasized evidence coming from Latin American countries.

Keywords: COVID-19, pregnancy, renin–angiotensin–aldosterone system, coronavirus in pregnancy, placenta

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INTRODUCTION

In December 2019, an unknown etiology outbreak of pneumonia was described in Wuhan, China. By January 2020, a new type of coronavirus was identified as the primary cause of these pneumonia cases. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) was identified as a beta coronavirus, the same subtype as its predecessors SARS-CoV and the Middle East Respiratory Syndrome (MERS-CoV). With the rapid spread of cases, the WHO declared a pandemic of coronavirus disease of 2019 (COVID-19) on March 11, 2020.

Severe Acute Respiratory Syndrome Coronavirus 2 causes essential alterations in the cardiovascular system beyond the initial damage to the respiratory system (Zheng et al., 2019). Alterations include the vascular endothelium-mediated recruitment of inflammatory leukocytes that contribute to tissue damage and cytokine release. These alterations are critical drivers of the acute respiratory distress syndrome and disseminated intravascular coagulation and COVID-19-associated cardiovascular complications.

During pregnancy, physiological adaptive changes in the immune, respiratory, cardiovascular, and coagulation systems could modulate COVID-19 presentation. As pregnant women are at high risk of complications and severe disease from infection with other coronaviruses, they were identified as a vulnerable group and were advised to take additional precautions as the COVID-19 pandemic unfolded. Accordingly, later evidence has shown that pregnant women seem to be associated with a greater susceptibility to contagion (Wastnedge et al., 2021), presenting more severe forms of the disease (Liu H. et al., 2020), or a high risk for pregnancy complications (di Mascio et al., 2020). In addition, data have confirmed SARS-CoV-2 vertical transmission, although short- and long-term sequels are still under investigation. Despite that, histological studies of placentas from SARS-CoV-2 positive pregnant women showed poor uterine-placental perfusion, with signs of placental infarcts, atheromas in the decidua vessels chorioangioma, and edema in placental villi (Mulvey et al., 2020; Shanes et al., 2020).

This review summarizes the current literature on SARS-CoV-2 virus infection in pregnancy. In addition, we describe pregnancy-associated adaptations in the angiotensin-converting enzyme 2 (ACE2), a protein identified as SARS-CoV-2 receptor, which could influence the presentation of COVID-19. This review particularly emphasized evidence coming from Latin American countries.

PATHOPHYSIOLOGY OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 INFECTION

Severe Acute Respiratory Syndrome Coronavirus 2 is an encapsulated positive single-stranded RNA virus, a coronavirus (Elias et al., 2021). The coronavirus family comprises numerous viruses with the capacity to infect several species (Channappanavar et al., 2014). So far, some of them, including coronaviruses 229E and NL63, display the capacity to affect humans, generating symptoms of a standard cold (Rabi et al., 2020). The comprising ability of these viruses to bind host cells requires a projection of the membrane structure, called "*spike*." This transmembrane structure is composed of a trimetric glycoprotein protuberance, composed of two subunits. The first one, S1, is the binding region to the host cell receptor and, S2 is the region where the virus and the host cellular membranes may fuse (Tang et al., 2020). Person-to-person transmission has been demonstrated through drops, contact, and aerosols spread, but less frequently due to fecal-oral and fomites transmission. The incubation period is around 5 days (5.79–6.97 days) (Elias et al., 2021). For diagnosis, the standard gold technique is the real-time PCR (RT-PCR), which detects the presence of SARS-CoV-2 virus RNA.

At the onset of COVID-19, the most common symptoms are fever, cough, and fatigue, while other symptoms include sputum production, headache, diarrhea, dyspnea, and lymphopenia (Rothan and Byrareddy, 2020). Once inside the host, SARS-CoV-2 activates the innate and adaptive immune responses and elicits a pronounced lymphopenia due to impaired lymphopoiesis and increased lymphocyte apoptosis (Wiersinga et al., 2020). Around 7–8 days after the onset of signs/symptoms, some individuals progress to a more clinically compromised condition and develop pneumonia with respiratory distress requiring hospitalization. In some cases, sepsis appears around days 9–10, and severe acute respiratory syndrome occurs on days 9–12. In some individuals, the start of mechanical ventilation and admission to the Intensive Care Unit (ICU) is required approximately at 10.5 days. Complications, such as heart or kidney injury, usually occur on day 15, secondary infection on day 17, and death or recovery on days 19–22 (Huang et al., 2020; Zhou F. et al., 2020). Some factors that seem to be associated with a higher risk of infection and severe disease are age (>70 years old), male sex, tobacco use, and presence of comorbidities, such as chronic diseases (Cai, 2020; Jin et al., 2020; Jordan et al., 2020; Zheng et al., 2020). Additionally, a high score on the Sequential Organ Failure Assessment (SOFA) and values >1 μg/ml for D-dimer have been associated with higher mortality (Zhou F. et al., 2020).

The mechanisms of SARS-CoV-2 invasion of the host system are illustrated in **Figure 1**. Briefly, the coronavirus virion has structural proteins: nucleocapsid (N), membrane (M), envelope (E), and spike (S) proteins. The entry steps of the viral particles—encompassing attachment to the host cell membrane and fusion—are mediated by the S glycoprotein. S protein is assembled as a homotrimer and is inserted in multiple copies into the virion membrane giving it its crown-like appearance. In addition, the furin-like proteases, transmembrane protease, serine 2 (TMPRSS2), and cathepsin L are involved in the virus invasion process. First, the S protein binds to the ACE2 receptor, after which the virus penetrates the host cells by endocytosis (S1 region) or membrane fusion (S2 region). Cleavage of the S1–S2 boundary is necessary for initiating the membrane-fusion process. After S1–S2 is cleaved, the S2 site activates the fusion process either by TMPRSS2 on the cell surface or by cathepsins in endosomes. Then, the fusion between viral and cellular membranes forms a pore through which viral RNA is released

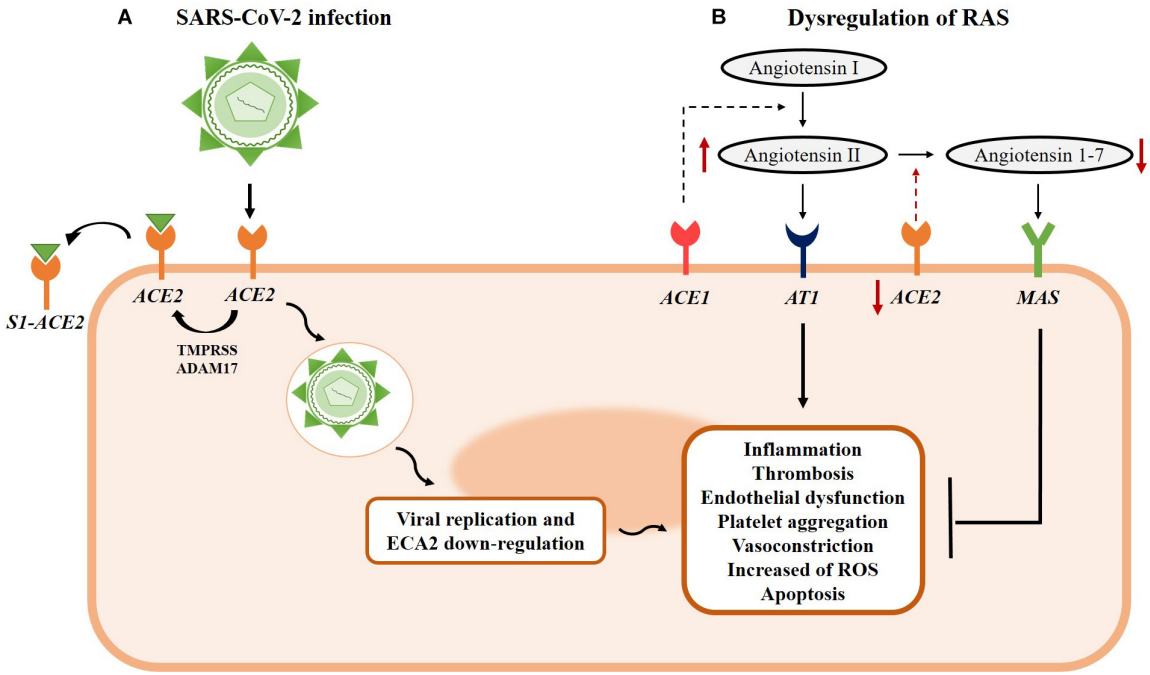

FIGURE 1 | Mechanism of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection and RAS system dysregulation. **(A)** SARS-CoV-2 uses the ACE2 receptor to enter human cells. Spike proteins can be cleaved by different host proteases to bind to ACE2 receptors. The S1-ACE2 interaction triggers the cleavage of ACE2 through transmembrane protease, serine 2 (TMPRSS) proteases or by ADAM17/TACE, resulting in the release of S1-ACE2 interaction from the host cells. The S1 angiotensin-converting enzyme 2 (ACE2) complex is followed by membrane fusion and subsequent viral endocytosis, which releases the viral genome into the cytoplasm, ACE2 downregulation, and pathological cellular effects. **(B)** The SARS-CoV-2 infection process contributes to RAS dysregulation due to ACE2 downregulation and, consequently, reduced conversion of Ang 1–7 from angiotensin II (Ang II) and increased levels of Ang II. The balance between ACE1 and ACE2 is necessary to maintain physiological conditions and the production levels of Ang II and Ang 1–7, respectively. The Ang II/AT1 interaction results in long-term exacerbated vasoconstriction, inflammation, thrombosis, and epithelial dysfunction. On the other hand, the Ang 1–7/MAS complex counteracts the harmful effects of Ang II by inducing vasodilation, anti-inflammatory effects, and tissue repair. In SARS-CoV-2 infection, the upregulation of Ang II leads to several long-term detrimental effects, while the lack of Ang 1–7 reduces the protective and counter-regulatory activities of the effects triggered by Ang II.

into the host cell cytoplasm for uncoating and replication (Zhou P. et al., 2020; Jackson et al., 2021).

Interestingly, the main characteristic of COVID-19 is endothelial cell damage (Evans et al., 2020). Thus, infection with SARS-CoV-2 reduces ACE2-mediated regulation of vascular tone and causes endothelial dysfunction at multiple levels, such as inflammatory activation, cytokine storm, leukocyte infiltration, increased permeability, thrombosis, platelet aggregation, vasoconstriction, reactive oxygen species (ROS) production, and apoptosis (Evans et al., 2020).

ANGIOTENSIN-CONVERTING ENZYME 2 AND THE IMPLICATION OF THE RENIN-ANGIOTENSIN SYSTEM ON CORONAVIRUS DISEASE 2019

Angiotensin-converting enzyme 2 is constitutively expressed in several tissues, such as the lung, heart, kidney, and blood vessels (Zou X. et al., 2020). Therefore, SARS-CoV-2 has a particular tropism for the pulmonary system, initiating pronounced respiratory symptoms (Jia et al., 2005). In addition, the renin-angiotensin system is a critical modulator of vascular function, as a key target of the SARS-CoV-2 infection (Watanabe et al., 2005; Fraga-Silva et al., 2013; Santos, 2014; Sanchis-Gomar et al., 2020). In particular, ACE2 is a pleiotropic peptidase that metabolizes Angiotensin II (Ang II) to Ang 1–7, two peptides with divergent physiological functions. Thus, while Ang II evokes vasoconstrictive, proliferative, and angiogenic effects, Ang 1–7 elicits anti-proliferative, anti-angiogenic, and vasodilator functions (Bharadwaj et al., 2011). Moreover, ACE2 has a protective effect on the endothelium and improves endothelial function, apparently mediated by Ang 1–7 production (Lovren et al., 2008; Fraga-Silva et al., 2013; **Figure 1**). The relevance of ACE2 has been confirmed in animals deficient in this enzyme. Those animals exhibit increased oxidative stress and pro-inflammatory cytokines, compromising cardiovascular function (Wang et al., 2020). Likewise, previous studies on the coronavirus-induced severe acute respiratory syndrome, sepsis, or acid aspiration-induced lung injury have shown that dysfunction of the renin-angiotensin system is involved in these conditions (Zhang et al., 2018; Li H. et al., 2020; Rezaei et al., 2021). Huang et al. (2014) reported that high plasma levels of Ang II were associated with the severity of the disease and also those high levels predicted fatal outcomes due to the H7N9 influenza

virus. In Kuba et al. (2005) described that the function of ACE2 is impaired by the binding of viral protein S, enhancing the Ang II circulating levels, and leading to hemodynamic alterations characterized by vasoconstriction.

The critical role of ACE2 in the COVID-19 pathophysiology raised speculations about ACE2 as a therapeutic target and the implications of the use of renin-angiotensin system inhibitors. Since ACE2 inhibitors and Ang II AT1 receptor antagonists have the potential to increase ACE2 expression, which may aggravate COVID-19, we recommend critical publications regarding the use of renin-angiotensin inhibitors and COVID-19 (Bavishi et al., 2020; Peiró and Moncada, 2020; Sanchis-Gomar et al., 2020; Sommerstein et al., 2020; Vaduganathaan et al., 2020). Yet the presence of the soluble and active isoform of ACE2 may be beneficial in COVID-19 patients since soluble ACE2 may compete with the membrane enzyme, limiting its role as a SARS-CoV-2 receptor, and decreasing the Ang II plasma levels (Khodarahmi et al., 2021). As a therapeutic target, the restoration of ACE2 through the administration of recombinant ACE2 may reverse the lung-injury process (Monteil et al., 2020, 2021; Siriwattananon et al., 2021; Zhang et al., 2021).

Therefore, several studies emphasize the critical role of ACE2 in viral infection, the clinical presentation of COVID-19, the potential use of its products (i.e., metabolites) as biomarkers. In addition, the use of drugs that modulate ACE2 has to be taken into account in the treatment and clinical evolution of COVID-19.

PREGNANCY IS A PHYSIOLOGICAL MODULATOR OF ANGIOTENSIN-CONVERTING ENZYME 2 EXPRESSION AND ACTIVITY

Two physiological conditions can modulate ACE2 levels and activity: aging and pregnancy (AlGhatrif, 2020; Huang et al., 2020; Ludvigsson, 2020; Zhou F. et al., 2020). Throughout gestation, a high expression of ACE2 in the human placenta, particularly in the decidua, the syncytiotrophoblast, and the villous stroma, may increase Ang 1–7 blood levels. Specifically, Liu D. et al. (2020) reported that ACE2 expression was upregulated between 6 and 16 weeks and downregulated in term human placentas. Plasmatic levels of ACE2 and Ang 1–7 are significantly augmented during pregnancy (Emanuele et al., 2002; Tamanna et al., 2020; Nobrega Cruz et al., 2021). On the other hand, Ang II was also increased in pregnancy (Emanuele et al., 2002). In this physiological scenario, it is believed that increased expression of ACE2 and blood levels of Ang 1–7 could be counteracting the increased stimulus of Ang II levels. Alternatively, the highest prevalence of a monomeric form of AT1, which is less sensitive to Ang II, may also reduce vascular sensitivity to Ang II. These changes would participate in the adaptive physiological mechanisms of the cardiovascular system during pregnancy, resulting in decreased peripheral vascular resistance and vasodilation of the maternal vasculature (Gant et al., 1973; AbdAlla et al., 2001; Levy et al., 2008; Marques et al., 2011; Pringle et al., 2011; Stettner et al., 2013), increased aldosterone and Ang II, promoting water and sodium retention, and increased blood volume (Scaife and Mohaupt, 2017). In addition, other critical factors modulated by the renin-angiotensin axis, increased during pregnancy, include the vasodilators prostaglandin E2, nitric oxide (Gant et al., 1980; Corthorn et al., 2006), and bradykinin (Knock and Poston, 1996). Therefore, pregnancy constitutes a physiological condition with major vascular adaptations characterized by reduced systemic vascular resistance that allows the homeostatic control of pregnancy-related hemodynamic changes, including increased cardiac output, expanded blood volume, and reduced blood pressure. Relevance of the renin-angiotensin axis in pregnancy is also remarkable in conditions in which deregulation of this axis impairs endothelial function and leads to pregnancy complications, such as hypertension or pre-eclampsia (Anton et al., 2008; Gilbert et al., 2008). Thus, it is not surprising that pregnant women constitute a potentially vulnerable population in the COVID-19 pandemic, with initial results indicating that the clinical response of pregnant women to COVID-19 could be related to physiological changes in expression levels of ACE2 and reduced sensitivity to Ang II (Anton et al., 2008; Gilbert et al., 2008; Liu D. et al., 2020; Mendoza et al., 2020).

CLINICAL PRESENTATION OF SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 INFECTION IN PREGNANT WOMEN

Some studies have reported that SARS-CoV-2 infection in pregnant women behaves similarly to the general population, contrary to what has been reported with other types of coronavirus infection in the past (Li N. et al., 2020; Yang et al., 2020; Fan et al., 2021). The disease manifests itself with typical symptoms and occurs with different degrees of severity, such as mild disease in 81–86% of cases, severe disease in 9.3–14%, and critical disease in 5%; values close to those reported in the general population (80, 15, and 5%, respectively) (Han et al., 2020). The most common symptoms were fever (65%), cough (60%), and shortness of breath or dyspnea (24%) (Han et al., 2020). Around 5% of mothers were admitted to the ICU; intubation was carried out in 35.87% of patients (Han et al., 2020). The rate of maternal death was <0.01%. Nevertheless, another study estimated the mortality rate in the pregnant population close to 2.7% and an ICU admission rate of 6–8% (Lambelet et al., 2020). In addition, Ellington et al. (2020) reported similar symptoms, with a greater risk of hospitalization, ICU intervention, and mechanical ventilation requirement, yet without an increased risk of death, in pregnant women when compared to non-pregnant women. It is noteworthy that, in this cohort, the group of pregnant women reported a higher frequency of comorbidities (chronic lung disease 22%, diabetes mellitus 15%, and cardiovascular disease 14%) when compared to non-pregnant women (Ellington et al., 2020).

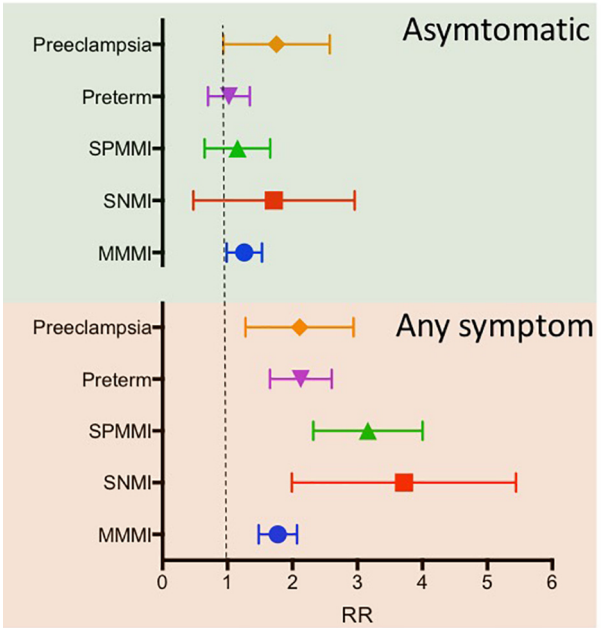

FIGURE 2 | Adjusted associations for maternal and perinatal outcomes among women with coronavirus disease 2019 (COVID-19) diagnosis according to symptom status. Adjusted models for the country, month entering study, maternal age, and history of maternal morbidity (such as diabetes, thyroid, and other endocrine disorders, cardiac disease, hypertension, chronic respiratory disease, kidney disease, malaria, or tuberculosis). The upper panel includes asymptomatic COVID-19 positive patients. At the same time, the bottom panel indicates COVID-19 patients with any symptoms. Pre-eclampsia includes a diagnosis of eclampsia and/or HELLP syndrome. HELLP, hemolysis, elevated liver enzymes, low platelet count; MMMI, maternal morbidity and mortality index; RR, relative risk; SNMI, severe neonatal morbidity index; SPMMI, severe perinatal morbidity and mortality index. Data extracted from Villar et al. (2021).

Severe Acute Respiratory Syndrome and MERS have been associated with miscarriage, intrauterine death, fetal growth restriction, and high case fatality rates (Wang et al., 2021). This linkage was also presented in the pandemic of SARS-CoV-2. Thus, infection in pregnant women was associated with a high risk of adverse pregnancy outcomes, such as intrauterine growth restriction, premature rupture of membranes, fetal distress, preterm delivery (Mullins et al., 2020), spontaneous abortion, and stillbirth (Della Gatta et al., 2020). In particular, Han et al. (2020) reported that premature delivery reached 25%. The rate of low birth weight (<2,500 g) was close to 31%, and neonatal intensive care unit (NICU) admission was 24%. Positive nasopharynx swabs or sputum from newborns was <0.01% (Han et al., 2020; **Figure 2**). More recently, a meta-analysis published by Wei et al. (2021) included 42 studies that involve 438,548 pregnant women. They found that COVID-19 was associated with pre-eclampsia [odds ratio (OR) 1.33], preterm birth (OR 1.82), and stillbirth (OR 2.11). In addition, they found that when compared with mild COVID-19, severe COVID-19 was strongly associated with pre-eclampsia (OR 4.16), preterm birth (OR 4.29), gestational diabetes (OR 1.99), and low birth weight (OR 1.89) (Wei et al., 2021). Another meta-analysis, including twenty-eight studies with 790,954 pregnant women, concluded that SARS-CoV-2 infection during pregnancy was associated with a 58% increased risk of pre-eclampsia. In addition, there was a statistically significant increase in the risk of pre-eclampsia with severe features (OR 1.76, $p < 0.05$), eclampsia (OR 1.97, $p < 0.05$), and Hemolysis, Elevated Liver enzymes, and Low Platelets (HELLP) syndrome (OR 2.10, $p < 0.05$) among pregnant women with SARS-CoV-2 infection, as compared to those without the infection (Papageorghiou et al., 2021). In addition, there is a high prevalence of cesarean delivery, whose main indication seems to be an underlying obstetric condition, such as pre-eclampsia, fetal distress, or premature rupture of membranes, and not the clinical condition of COVID-19 in pregnant women (Della Gatta et al., 2020; Trad et al., 2020).

Conversely, other studies reported that SARS-CoV-2 infection during the first trimester of pregnancy does not seem to predispose to early pregnancy loss (Cosma et al., 2020; Rotshenker-Olshinka et al., 2020; Bortoletto et al., 2021). In addition, Rizzo et al. (2021) reported that SARS-CoV-2 infection did not increase the risk of developing fetal growth restriction. At the same time, Meyer R. et al. (2021) showed a decrease in preterm delivery in pregnant women in Israel, possibly due to the reduction of iatrogenic preterm births, avoidance of infections, or reduced stress levels related to the lockdown policy (Meyer R. et al., 2021). However, the question about the differentiation between spontaneous or iatrogenic preterm delivery remains to be elucidated (Della Gatta et al., 2020). Moreover, there was no association between abnormal umbilical artery Doppler results, defined as a composite of increased S/D ratio, absent end-diastolic velocity, reversed end-diastolic velocity, and COVID-19 infection in growth-restricted pregnancies (Ona et al., 2021).

Differences in these reports depend on several factors. First, we propose that it is relevant to consider that a significant percentage of infected pregnant women could be asymptomatic or become undiagnosed. Remarking this issue, Sutton et al. (2020) reported that in a screening testing of COVID-19 in 215 pregnant women in New York (United States), 211 women were asymptomatic, but 13.7% tested positive to SARS-CoV-2. Thus, the prevalence of COVID-19 in pregnant women may be underestimated and therefore the associated perinatal complications (Lambelet et al., 2020; Sutton et al., 2020).

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 IN LATIN AMERICA

There are several publications about the SARS-CoV-2 pandemic in Latin America. Most of the articles are reports of a few cases in local hospitals. However, the Iberoamerican Society of Neonatology (SABEN) recruited women with SARS-CoV-2 infection to provide knowledge and experiences on perinatal COVID-19 in Latin America (Sola et al., 2020). Their results described 86 pregnant women with COVID-19 from 11 units of 7 countries: Argentina, Colombia, Ecuador, Equatorial Guinea, Honduras, Peru, and the Dominican Republic. Of these, 68%

women were asymptomatic for COVID-19, and 32% women exhibited symptoms. In total, 89% of symptomatic women had mild symptoms or signs, while 3.5% women had severe respiratory symptoms. Six women were admitted to intensive care, and no woman died. In addition, 94% were term, and 6% were preterm pregnancies. The swab result was positive in 7% of the newborns, with no causalities. Nevertheless, another study that includes 40 obstetric patients diagnosed with COVID-19 from four countries (Peru, Colombia, Bolivia, and Paraguay) reported a maternal mortality rate of 15% (six cases) and a perinatal mortality rate of 2.5% (one case). Associated pathologies included severe pre-eclampsia (25%), HELLP (5%), and gestational hypertension (12.5%), while ten patients received invasive mechanical ventilation since admission to ICU (25%) (Viruez-Soto et al., 2021; **Figure 3**).

In Hispanic women, obesity, advanced maternal age, medical comorbidities, and antepartum admissions related to COVID-19 have been reported as risk factors associated with adverse maternal and neonatal outcomes (Brandt et al., 2020). In Brazil, a report identified 20 COVID-19-related maternal deaths. Symptoms onset was reported during pregnancy for 12 cases, post-partum for three cases, and during the cesarean section for one case (missing data for four). In 16 cases, death occurred in the post-partum period. At least one comorbidity or risk factor was present in 11 cases (missing data for four). Asthma was the most common risk factor (5/11) (Takemoto et al., 2020).

Although Latin America has one of the highest COVID-19 death rates in the world (Editorial The Lancet, 2020), the data worldwide do not show a severe impact on maternal and fetal health (Sánchez-Duque et al., 2020). However, it is worth mentioning that only severe and critically ill patients have been tested for COVID-19, so the number of infected people (including pregnant and post-partum women) is more likely underestimated (Takemoto et al., 2020).

PLACENTAL INFECTION BY SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 AND POTENTIAL PATHOPHYSIOLOGICAL MECHANISM

There is a significant concern regarding eventual transplacental transmission, infection at delivery or through breast milk, and the general care measures for pregnant women and neonates are being systematized. Indeed, convincing pieces of evidence have detected SARS-CoV-2 in the human placenta (Algarroba et al., 2020a; Baud et al., 2020; Best Rocha et al., 2020; Chen et al., 2020; Fenizia et al., 2020; Ferraiolo et al., 2020; Hecht et al., 2020; Hosier et al., 2020; Kirtsman et al., 2020; Kulkarni et al., 2020; Patanè et al., 2020; Richtmann et al., 2020; Smithgall et al., 2020; Vivanti et al., 2020; Hsu et al., 2021; Marinho et al., 2021). Regarding localization of SARS-CoV-2 in the placenta, most of the studies with positive results detected the presence of the virus in the syncytiotrophoblast (Best Rocha et al., 2020; Hecht et al., 2020; Hosier et al., 2020; Patanè et al., 2020; Sisman et al., 2020; Vivanti et al., 2020) and only the study of Hsu et al. (2021) detected the presence of SARS-CoV-2 in the fetal endothelium. Despite that, other studies did not detect SARS-CoV-2 in the placenta (Edlow et al., 2020; Flores-Pliego et al., 2021; Halici-Ozturk et al., 2021; Levitan et al., 2021; **Table 1**). Therefore, although data are not consistent, there may be vertical transmission in some cases.

Interestingly, there is also evidence for *in utero* transmission of SARS-CoV-2 infection. For instance, detection of SARS-CoV-2 virus in nasopharyngeal samples of newborns and anti-SARS-CoV-2 antibodies detected in the umbilical cord blood in at least one case were reported (Fenizia et al., 2020). In addition, Sisman et al. (2020) presented a preterm infant with placental SARS-CoV-2 infection and positive nasopharyngeal testing at 24 and 48 h of life, who developed fever and mild respiratory disease on the second day of life.

Whether ACE2 and TMPRSS2 are entry mechanisms for SARS-CoV-2 in the placenta remains unclear, although both proteins are expressed in the trophoblast and fetal endothelium. Thus, ACE2 was detected in syncytiotrophoblast (Edlow et al., 2020; Hecht et al., 2020; Taglauer et al., 2020; Lu-Culligan et al., 2021) and endothelium of umbilical arteries (Valdés et al., 2006). Nevertheless, a study conducted in isolated cells from the placenta shows that the co-expression of ACE2 and TMPRSS2 is very low in syncytiotrophoblast, cytotrophoblast, and extravillous trophoblast, especially when it is compared with the expression of cytomegalovirus and Zika virus receptors. These pathogens have been proven to cross the placenta and generate vertical transmission to the fetus (Pique-Regi et al., 2020). In addition, Li M. et al. (2020) reported high expression of ACE2 and TMPRSS2 in syncytiotrophoblast and cytotrophoblast in the single-cell evaluation of trophoblasts in the human placenta. Despite this evidence, it is necessary to investigate whether the expression of both proteins in the placenta may be affected in pregnancy complicated by SARS-CoV-2 infection since a weak expression of TMPRSS2 in the villous endothelium of SARS-CoV-2 positive pregnant women was reported (Edlow et al., 2020; Hecht et al., 2020).

Additionally, fetal sex could affect the placental expression of ACE2. It has been reported that the male sex is associated with a higher ACE2 gene expression (Chlamydas et al., 2021). Moreover, it is known that the ACE2 gene escapes X inactivation (Tukiainen et al., 2017), *in vitro* study shows that 17β-estradiol, a primarily female sex steroid, can downregulate the ACE2 gene expression in non-pregnant women (Stelzig et al., 2020), providing another hypothesis for the sex-based expression differences of this gene. However, there is no information about sex-dimorphism in the placental expression of ACE2 in pregnant women with COVID-19.

On the other hand, detecting SARS-CoV-2 in placentas from fetal death cases alerted the possible occurrence of placental dysfunction associated with the maternal medical condition (Baud et al., 2020; Hosier et al., 2020; Richtmann et al., 2020). These studies suggest that early infection (first or second trimester of pregnancy) could generate a state of more significant deterioration in the placental function associated with exacerbated inflammation, negatively impacting fetal wellbeing and development. Supporting this pathophysiological explanation, a recent case report from

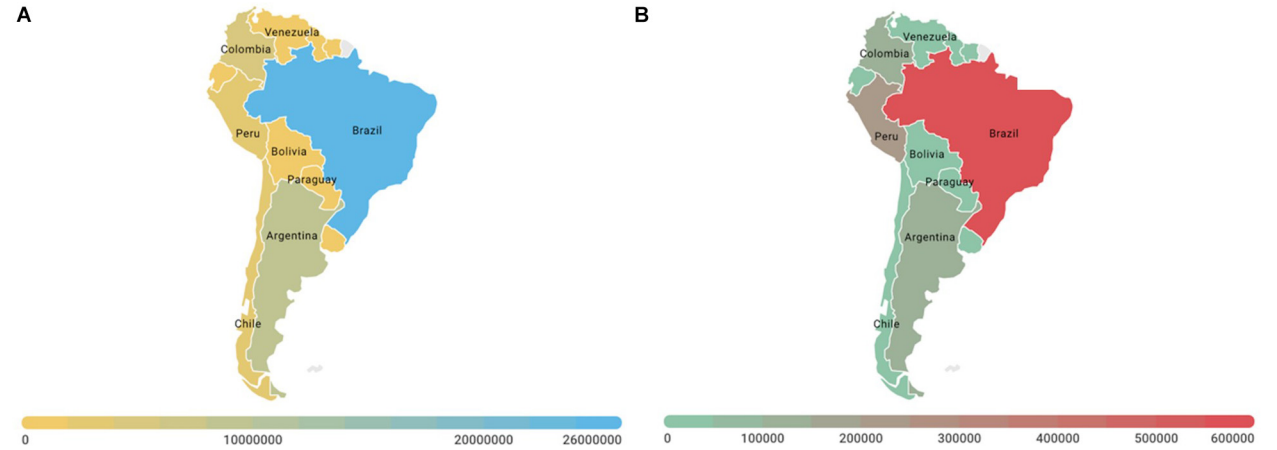

FIGURE 3 | Morbi-mortality associated with coronavirus disease (COVID-19) in Latin America. **(A)** Total confirmed cases and **(B)** total mortality in Latin American countries at February 3, 2022. Brazil is the Latin American country affected the most by the COVID-19 pandemic. As of February 2022, the country had reported over 26 million cases, and 630 thousand of deaths. Source WHO; CDC; ECDC; NHC; DXY.cn; Johns Hopkins University.

TABLE 1 | Studies that assess the detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) in placenta around the world indicate the technique used and the state of symptoms.

Method of detection of SARS-CoV-2 in placenta				Presence of COVID-19 symptoms		Total cases	Country	References
qPCR	IHC	ISH	EM	+	−			
Neg	ND	ND	ND	1		1	Turkey	Kalafat et al., 2020
Neg	ND	ND	ND	1		1	China	Peng et al., 2020
Pos(1)	ND	ND	ND	11		11	United States	Penfield et al., 2020
ND	ND	ND	Pos	1		1	United States	Algarroba et al., 2020b
ND	Pos(1)	Pos(2)	ND	ND	ND	19	United States	Hecht et al., 2020
ND	Pos	ND	ND		1	1	United States	Hsu et al., 2021
Pos	ND	ND	ND	1		1	India	Kulkarni et al., 2020
Pos	ND	ND	ND	4	1	5	Brazil	Richtmann et al., 2020
ND	Neg	Neg	ND	50	26	76	United States	Smithgall et al., 2020
Pos	Pos	ND	ND	ND	ND	1	United States	Vivanti et al., 2020
ND	Pos	ND	Pos	1		1	United States	Sisman et al., 2020
ND	Pos	Pos	ND	1	1	2	United States	Best Rocha et al., 2020
Pos	ND	ND	ND		1	1	Italy	Ferraiolo et al., 2020
Pos	Pos	Pos	Pos	1		1	United States	Hosier et al., 2020
ND	Pos	Pos(2)	ND	3		3	Italy	Patanè et al., 2020
Pos	ND	ND	ND	3		3	China	Chen et al., 2020
Pos	ND	ND	ND	1		1	Switzerland	Baud et al., 2020
Pos	ND	ND	ND	1		1	Canada	Kirtsman et al., 2020
Pos (2)	ND	ND	ND	31		31	Italy	Fenizia et al., 2020
ND	ND	Neg	ND	15	32	47	United States	Edlow et al., 2020
Neg	ND	ND	ND	11		11	Mexico	Flores-Pliego et al., 2021
Neg	ND	ND	ND	5	19	24	Turkey	Halici-Ozturk et al., 2021
Pos	Pos	Pos	Pos	1		1	Mexico	Valdespino-Vázquez et al., 2021

qPCR, real-time PCR; IHC, immunohistochemistry; ISH, In situ hybridization; EM, electron microscopy; pos, positive; Neg: negative; ND, not determined.

Brazil showed an association of fetal death at 34 weeks of gestation with histopathological alterations in the placenta. The findings are consistent with intense acute placenta vascular malperfusion, with the detection of SARS-CoV-2 in the placenta, umbilical cord, and fetal tissues (lung, heart, and brain, among others). Furthermore, the patient had mild symptoms of COVID-19 but, at the time of fetal death, had pro-inflammatory and pro-coagulant syndrome characterized by higher levels of interleukins, ferritin, and D-dimer (Marinho et al., 2021). Therefore, further research is needed to elucidate the

involvement of placental dysfunction in COVID-19 cases and its perinatal consequences.

PLACENTAL VASCULAR DISORDERS ASSOCIATED WITH SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2

Regarding the vascular alterations of the placenta, histological studies of placentas from SARS-CoV-2 positive pregnant women showed poor uterine-placental perfusion, with signs of placental infarcts, atheromas in decidua vessels, chorioangioma, and edema in the placental villi (Mulvey et al., 2020; Shanes et al., 2020). Those studies also associated vascular thrombosis in the chorionic plate and decreased capillaries density in the chorionic villi in placentas with SARS-CoV-2 infection. Moreover, Baergen and Heller (2020) described that this viral infection was associated with a high risk of fetal vessel thrombosis and reduced vascularization in the placental microcirculation. Similarly, Patberg et al. (2020) showed that SARS-CoV-2 infection is associated with placental signs of reduced perfusion and villitis of unknown etiology. Another study confirmed these findings, including asymptomatic or mildly symptomatic SARS-CoV-2 positive pregnant women, showing evidence of fetal vascular malperfusion (FVM): chorioangiosis, intramural fibrin deposition, and vascular ectasia. Additionally, perivillous fibrin deposition was also significantly higher in placental histopathology (Jaiswal et al., 2021). These results agree with Meyer J. et al.'s (2021) findings showing that 77% of placentas infected with SARS-CoV-2 showed one or more features of maternal vascular malperfusion (MVM). Therefore, growing pieces of evidence, with some exceptions (Zhang et al., 2020; Levitan et al., 2021; Lu-Culligan et al., 2021), have shown placental vascular disorders in pregnant women with COVID-19.

Whether the severity of COVID-19 in pregnancy is related to placental alterations is under investigation. For instance, high trophoblast necrosis was found in pregnant women who required respiratory support or intubation for COVID-19 when compared with non-hypoxic patients (Meyer J. et al., 2021). Moreover, Edlow et al. (2020) found that placental lesions associated with MVM were increased with the severity of COVID-19 in pregnant women. Remarkably, in patients with COVID-19 admitted to the ICU, increased levels of von Willebrand Factor (vWF) antigen and P-selectin in plasma were detected, indicating endothelial dysfunction (Goshua et al., 2020). In addition, in the placenta of pregnant women with severe COVID-19, higher expression of vWF was associated with lower claudin-5 and vascular endothelial (VE-cadherin) in the endothelium from decidua and chorionic villi (Flores-Pliego et al., 2021). These findings suggest that COVID-19 induces endothelial cell injury in the placenta, probably affecting the endothelial barrier, the anti-thrombotic capacity of the endothelium, and the overall function of placental vessels. Compromised placental function, placental hypoxia, and a hypercoagulable state are probably related to the severity of the infection (Ng et al., 2006; Baergen and Heller, 2020; Shanes et al., 2020; Marinho et al., 2021; Meyer J. et al., 2021; Poisson and Pierone, 2021; **Figure 4**).

In addition, SARS-CoV-2 positive mothers showed higher levels of inflammatory markers. Thus, Fenizia et al. (2020) showed significant increases in inflammatory markers (including interleukin 1β or interleukin 6) in both placenta and plasma of two women whose placenta was tested positive for SARS-CoV-2. Accordingly, it was previously reported that the presence of SARS-CoV-2 in the placenta generated an increased inflammatory response in the intervillous space (histiocytic intervillositis) and chorionic villi (villositis), with the presence of macrophages (CD68) and T lymphocytes (CD3) in the intervillous space (Hecht et al., 2020; Hosier et al., 2020; Kirtsman et al., 2020; Patanè et al., 2020; Sisman et al., 2020; Smithgall et al., 2020; Vivanti et al., 2020; Hsu et al., 2021). Transcriptome analysis of placentas from pregnant women with COVID-19 showed increased genes associated with immune response. Specifically, Lu-Culligan et al. (2021) found a marked increase of pro-inflammatory genes and chemokines in both immune and non-immune cell types in placentas from COVID-19 cases. In addition, single-cell transcriptome analysis revealed significant enrichment of genes encoding cytotoxic proteins in natural killer (NK) cells, associated with upregulation of the activation marker CD69 in T-cells, increased expression of interferon-induced protein ISG15, and the regulators of nuclear factor-κB (NFκB) pathway in endothelial cells (Lu-Culligan et al., 2021).

Despite these findings, the potential implications of vascular alterations of the placenta in COVID-19 cases and fetal development are not entirely understood (Ahlberg et al., 2020; Díaz-Corvillón et al., 2020; Khalil et al., 2020; Shanes et al., 2020). Therefore, there is a demanding necessity to continue gathering information, aiming to elucidate the potential implication of impaired placental environment observed in women with COVID-19 in contributing to adverse perinatal outcomes, particularly in periods of the high spread of the pandemic.

THE IMPACT OF CORONAVIRUS DISEASE 2019 VACCINE ON PREGNANCY

Pregnant women have traditionally been excluded from vaccine trials. Without appropriate evidence about safety and efficacy during pregnancy, they have previously been denied the opportunity to receive vaccines that would have protected them and their offspring. This situation has also been present in the context of the COVID-19 pandemic, and pregnant women, their providers, and health policymakers would have to make unnecessarily tricky decisions because of inadequate evidence about vaccine use in pregnancy. This would lead to less vaccine use and its afforded protections in this population (Beigi et al., 2021). In the V-SAVE pregnancy registry with 3,958 participants who received messenger RNA (mRNA) COVID-19 vaccines, 827 had a completed pregnancy, of which 115 (13.9%) resulted in a pregnancy loss and 712 (86.1%) resulted in a live birth (mostly among pregnant women vaccinated in the third trimester). Adverse neonatal outcomes included preterm

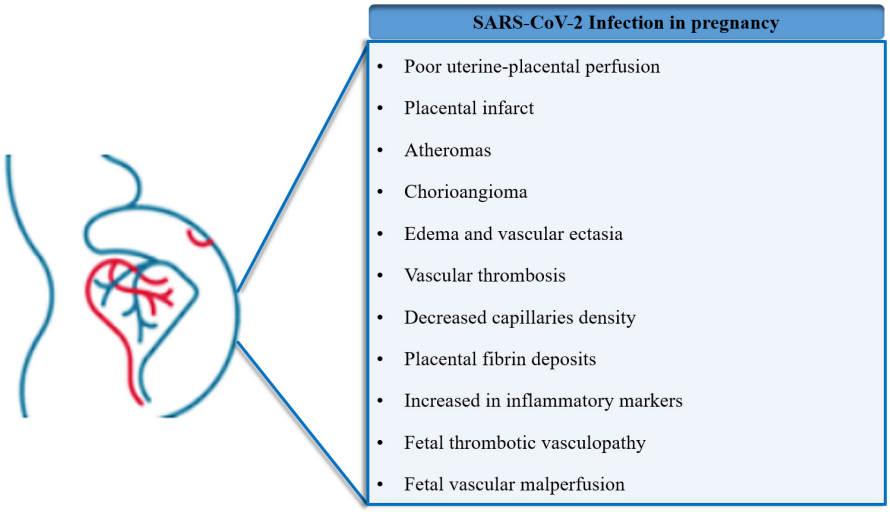

FIGURE 4 | Major placental and gestational disorders associated with Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Pregnant women constitute a population group with special conditions due to the imminent risks to the mother and fetus. Recent evidence has shown that SARS-CoV-2 infection during pregnancy results in distinct physiological alterations, affecting placental function and distinct gestational parameters.

birth (in 9.4%) and small size for gestational age (in 3.2%), and no neonatal deaths were observed. Although not directly comparable, calculated proportions of adverse pregnancy and neonatal outcomes in persons vaccinated against COVID-19 who had a completed pregnancy were similar to incidences reported in studies involving pregnant women that were conducted before the pandemic (Shimabukuro et al., 2021). On the other hand, a recent study in 24,288 singleton pregnancies showed no evident differences, in terms of adverse neonatal and early infant outcomes, between newborns of women who received BNT162b2 mRNA vaccination during pregnancy vs. those of women who were not vaccinated. This study contributes to current evidence in establishing the safety of prenatal vaccine exposure to newborns. However, the interpretation of study findings is limited by the observational design (Goldshtein et al., 2022).

Clinical trials demonstrate that vaccination effectively prevents severity and symptomatic COVID-19 in non-pregnant persons. To highlight, the risks of administering COVID-19 vaccines during pregnancy appear to be minimal. Commonly reported side effects are short-term injection site pain, headache, fever, myalgia, arthralgia, chills, and nausea (National Center for Immunization and Respiratory Diseases, 2021). Moreover, the rate of serious adverse effects has been relatively low (National Center for Immunization and Respiratory Diseases, 2021). In addition, chemical components of the vaccines are not specifically contraindicated in pregnancy (National Center for Immunization and Respiratory Diseases, 2021). About the recommendations, the American College of Obstetrics and Gynecology states that in the absence of data showing that vaccines are contraindicated, then pregnant patients should be immunized (American College of Obstetricians and Gynecologists' Immunization Infectious Disease and Public Health Preparedness Expert Work Group et al., 2021). The Center for Disease Control has taken a similar position, declaring that the only absolute contraindication to vaccination is an allergy to vaccine components (National Center for Immunization and Respiratory Diseases, 2021). However, the World Health Organization has more reserved recommendations, indicating that vaccination is only indicated in pregnant women who are at high risk for exposure to COVID-19 (healthcare workers, or those with comorbidities that might make disease more severe) (Chavan et al., 2021; World Health Organization, 2021).

CONCLUDING REMARKS

In this manuscript, we have discussed the vulnerability of the pregnant population to COVID-19 infection. This manuscript also described many uncertainties; however, they are not the only ones in this field. For example, the real incidence of COVID-19 in pregnant women is a fundamental question that needs clarification. In addition, whether COVID-19 is associated with a greater risk of severe disease and perinatal complications requires confirmatory population studies. This information would help to generate appropriate public health policies for this particular population.

In addition, we also have remarked that infected placenta with SARS-CoV-2 showed alterations related to inflammatory processes associated with damages to the vascular network. Whether these placental alterations might explain the adverse perinatal outcomes in women with COVID-19 requires confirmatory studies. In addition, although there is vertical transmission in some cases, vertical transmission data are not consistent. We encourage future research to elucidate whether SARS-CoV-2 infection affects fetal programming, as well as the future health of both mother and offspring.

Concerning the vaccine, pregnant women and physicians need to use the limited available data to weigh the benefits and risks of the COVID-19 vaccine during pregnancy, considering the patient's specific risk of SARS-CoV-2 exposure. Currently, there is the absence of evidence that supports pregnancy as a contraindication, and it seems that the benefits of receiving the vaccine far outweigh the unlikely potential harms. However, estimates of global vaccination among pregnant women are yet unknown. Moreover, we remark inequities in the access to the SARS-CoV-2 vaccines worldwide, but, in particular, in Latin America. For example, only 56% of the Latin American people have been vaccinated up to December 2021 (PAHO, 2021), with a significant difference among countries (PAHO, 2022). In addition, it is necessary to delve into how the pandemic has impacted the care of pregnant women in general since substantial and heterogeneous modifications have been reported in maternity services (Jardine et al., 2021).

In conclusion, the available information highlights the greater vulnerability of pregnant women in the context of a pandemic. However, more studies are required to better understand the potential impact of the pandemic on pregnant women, especially in Latin American nations.

AUTHOR CONTRIBUTIONS

PA-R, MG, and RG-R conceived, designed, planned, and supervised the manuscript. PA-R, MG, and CE critically review the manuscript and generate the final published version. LQ-A, FG, RA, CE, and AD wrote the manuscript. All authors provided critical feedback and approved the final version.

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REVIEW
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Pathophysiological Role of Genetic Factors Associated With Gestational Diabetes Mellitus

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Gestational Diabetes Mellitus (GDM) is a highly prevalent maternal pathology characterized by maternal glucose intolerance during pregnancy that is, associated with severe complications for both mother and offspring. Several risk factors have been related to GDM; one of the most important among them is genetic predisposition. Numerous single nucleotide polymorphisms (SNPs) in genes that act at different levels on various tissues, could cause changes in the expression levels and activity of proteins, which result in glucose and insulin metabolism dysfunction. In this review, we describe various SNPs; which according to literature, increase the risk of developing GDM. These SNPs include: (1) those associated with transcription factors that regulate insulin production and excretion, such as rs7903146 (*TCF7L2*) and rs5015480 (*HHEX*); (2) others that cause a decrease in protective hormones against insulin resistance such as rs2241766 (*ADIPOQ*) and rs6257 (*SHBG*); (3) SNPs that cause modifications in membrane proteins, generating dysfunction in insulin signaling or cell transport in the case of rs5443 (*GNB3*) and rs2237892 (*KCNQ1*); (4) those associated with enzymes such as rs225014 (*DIO2*) and rs9939609 (*FTO*) which cause an impaired metabolism, resulting in an insulin resistance state; and (5) other polymorphisms, those are associated with growth factors such as rs2146323 (*VEGFA*) and rs755622 (*MIF*) which could cause changes in the expression levels of these proteins, producing endothelial dysfunction and an increase of pro-inflammatory cytokines, characteristic on GDM. While the pathophysiological mechanism is unclear, this review describes various potential effects of these polymorphisms on the predisposition to develop GDM.

Keywords: gestational diabetes mellitus, single nucleotide polymorphism, insulin resistance, genetic risk factors, insulin signaling dysfunction

HIGHLIGHTS

1) Several SNPs cause predisposition to GDM.
2) SNPs associated with GDM mainly affect endocrine pancreas function and adipose tissue response to insulin.
3) Physiopathology induced by these SNPs could explain GDM development.

1 INTRODUCTION

The American Diabetes Association (ADA) defines gestational diabetes mellitus (GDM) as a type of diabetes diagnosed at the second or third trimester of pregnancy in a mother not diagnosed with pregestational diabetes (ADA, 2020). In recent years, it has been observed that the incidence of this pathology is increasing along with obesity and type 2 diabetes mellitus (T2DM) (Coustan, 2013; ADA, 2019). It is estimated that the worldwide prevalence of GDM varies between 1.7 and 11.7% (Griffin et al., 2000; Schneider et al., 2012). This considerable variation is due to differences among the populations and diagnostic criteria used in each country. It has been estimated that countries with higher incidence of GDM are those of Middle East and North Africa with 12.9%, followed by Southeast Asia (11.7%) (Zhu and Zhang, 2016). In developed countries such as the United States, Australia, Canada and the United Kingdom, its prevalence is less than 6% (Zhao et al., 2016). South American countries show high GDM prevalence: about 15% of pregnant women from Peru and Chile have been diagnosed with GDM in the last 20 years (Belmar et al., 2004; Huidobro et al., 2004; Larrabure-Torrealva et al., 2018; Garmendia et al., 2019).

One of the proposed risk factors for GDM is obesity. In fact, GDM women usually have a body mass index (BMI) higher or equal to 25 kg/m^2 (Shah et al., 2011). An increase in proinflammatory cytokines have been reported in obese pregnant women affected with GDM (Kinalski et al., 2005; Kuzmicki et al., 2008; Pantham et al., 2015). This pro-inflammatory state stimulates the synthesis of xanthurenic acid, which has been associated with the development of T2DM, prediabetes, and GDM (Bennink and Schreurs, 1975; Oxenkrug, 2015; Law and Zhang, 2017). Moreover, in GDM pregnant women there is a supraphysiological insulin resistance state induced in part by pro-inflammatory cytokines (Sonagra et al., 2014). For that reason, GDM pregnancies are associated with high HOMA-IR index values (Wang et al., 2018).

The main maternal outcomes of GDM are hyperglycemia, GDM in future pregnancies, future development of T2DM (Griffin et al., 2000; Ben-Haroush et al., 2004), obesity, and preeclampsia (Coustan, 2013; Kampmann et al., 2015). A study by the HAPO Study Cooperative Research Group demonstrated that in GDM pregnancies, the main fetal outcomes are macrosomia, neonatal hyperinsulinemia, caesarean section, and neonatal hypoglycemia (HAPO Study Cooperative Research Group, 2002; HAPO Study Cooperative Research Group, 2008). Furthermore, newborns from GDM pregnancies have a high risk of developing T2DM and obesity in the long-term (Dabelea et al., 2000).

Maternal and fetal outcomes are associated with modifiable and non-modifiable factors (Shaat et al., 2007). One of these non-modifiable factors is genetics. In this line, diverse Genome-Wide Association Studies (GWAS) have shown that genetic variables of the single nucleotide polymorphisms (SNPs) type associated with T2DM have also been related with high predisposition to GDM, which has been studied in diverse populations (Kwak et al., 2012; Huerta-Chagoya et al., 2015; Lowe et al., 2016). This association has been proposed because both GDM and T2DM are associated with similar pathophysiology mechanisms, including insulin resistance and a chronic inflammatory state (Zajdenverg and Negrato, 2017). Furthermore, GDM increases the risk of progression to T2DM; nevertheless, the magnitude of this effect is variable in different populations (Plows et al., 2018; Vounzoulaki et al., 2020). Unlike T2DM, GDM is triggered by placental and maternal hormones that cause a transitory insulin resistance that in most cases disappears after pregnancy, and only affects pregnant women (Mack and Tomich, 2017).

The positive associations of GDM and genetic variations is not clear in all cases (Anghebem-Oliveira et al., 2017), and the mechanisms by which these could contribute to GDM development have not been fully described. Therefore, this review summarizes the main genetic variants that have been described for GDM, emphasizing their potential pathophysiological mechanisms on the generation of GDM.

2 GENETIC FACTORS ASSOCIATED WITH GESTATIONAL DIABETES MELLITUS AND ITS PATHOPHYSIOLOGICAL MECHANISM OVER THE GDM ETIOLOGY

GDM etiology could be associated with genetic variations that are related to T2DM development (Buchanan and Xiang, 2005; Gong et al., 2016). GWAS have identified numerous loci associated with the risk of GDM (Dalfrà et al., 2020). In this review, we classify different SNPs that have been associated with T2DM and GDM, according to the protein they encode (**Table 1**).

The presence of genetic variants can result in changes in the expression and function of the encoded protein, affecting diverse physiological actions. For this reason, polymorphisms can be clinically relevant for various pathologies (Ramírez-Bello and Jiménez-Morales, 2017). In the following section, we focus on explaining the pathophysiological mechanisms caused by the main aforementioned genetic factors.

2.1 Polymorphisms Associated With Transcription Factors

2.1.1 *TCF7L2* Genetic Variants Associated to GDM

The transcription factor 7-like 2 (*TCF7L2*) gene is located on chromosome 10 and encodes for a nuclear protein that participates in gene expression regulation involved in the fusion of insulin secreting granules in pancreatic beta cells (da Silva et al., 2009). In a multiracial study, carriers of the T allele of SNP rs7903146 (risk-allele) showed an increased risk of T2DM

TABLE 1 | Single nucleotide polymorphisms associated with gestational diabetes mellitus.

Gene	SNP	Number of Participants	Population	Genetic Variant	OR (95%CI)	References
ADIPOQ	rs2241766	135 controls and 135 GDM	Chinese	TG + GG vs. TT G allele	1.67 (1.03–2.70) 1.55 (1.08–2.23)	Feng et al. (2019)
CDKAL1	rs7754840	2025 controls and 1399 GDM	Korean	C allele	1.52 (1.37–1.68)	Kwak et al. (2012)
	rs7748720	315 controls and 319 GDM	Chinese	AA+ GA vs. GG	1.46 (1.01–2.10)	Wang et al. (2019)
	rs6938256			GG + AG vs. AA	0.58 (0.42–0.81)	
DIO2	rs225014	516 controls and 1057 T2DM	Brazilian	C allele	1.18 (1.03–1.36)	Dora et al. (2010)
FTO	rs9939609	7229 controls and 3636 GDM	Multi-ethnic (Meta-analysis)	AA vs. TT AA vs. AT + TT A vs. T	1.33 (1.05–1.68) 1.31 (1.07–1.61) 1.12 (1.01–1.28)	Lin et al. (2018)
	rs1121980	1021 controls and 964 GDM	Chinese	A allele	0.79 (0.67–0.94)	Cao et al. (2020)
GLIS3	rs10814916	6086 controls and 2636 GDM	American and Danish	C allele	1.16 (1.08–1.24)	Ding et al. (2018)
	rs7041847			A allele	1.13 (1.05–1.20)	
GNB3	rs5443	130 controls and 120 GDM	Chinese	CT + TT vs. CC	1.91 (1.05–3.46)	Feng et al. (2019)
GPSM1	rs11787792	6086 controls and 2636 GDM	American and Danish	A allele	0.87 (0.80–0.94)	Ding et al. (2018)
HHEX	rs5015480	204 GDM and 207 NGT	Polish	C allele	1.40 (1.05–1.87)	Tarnowski et al. (2017)
		18 studies (18227 GDM and 30366 controls)	Multi-ethnic (Meta-analysis)		1.16 (1.06–1.26)	Li et al. (2012)
		4 studies (3513 controls and 1651 GDM)	Multi-ethnic (Meta-analysis)		1.24 (1.12–1.38)	Wang et al. (2020)
HNF1A	rs7957197	6086 controls and 2636 GDM	American and Danish	T allele	1.22 (1.12–1.33)	Ding et al. (2018)
KCNQ1	rs2237892	453 controls and 562 GDM	Chinese	C allele	2.19 (1.36–3.54)	Ao et al. (2015)
	rs163182	1021 controls and 964 GDM	Chinese	C allele	0.84 (0.73–0.96)	Cao et al. (2020)
MC4R	rs12970134	1021 controls and 964 GDM	Chinese	A allele	1.25 (1.07–1.46)	Cao et al. (2020)
	rs2229616	676 controls and 753 GDM	Chinese	T allele	1.62 (1.05–2.50)	Shen et al. (2020)
MIF	rs755622	485 controls and 430 GDM	Chinese	C allele	1.59 (1.28–1.98)	Li et al. (2016)
MTNR1B	rs10830962	2025 controls and 1399 GDM	Korean	G allele	1.45 (1.32–1.61)	Kwak et al. (2012)
	rs10830963	6086 controls and 2636 GDM	American and Danish	G allele	1.27 (1.18–1.37)	Ding et al. (2018)
	rs1387153			T allele	1.17 (1.09–1.26)	
	rs10830963	676 controls and 753 GDM	Chinese	G allele	1.36 (1.17–1.59)	Shen et al. (2020)
	rs1387153			T allele	1.40 (1.20–1.63)	
	rs1447352			G allele	0.82 (0.69–0.97)	
	rs2166706			C allele	1.36 (1.17–1.59)	
	rs4753426			T allele	0.84 (0.71–0.99)	
PROX1	rs340841	1021 controls and 964 GDM	Chinese	T allele	1.22 (1.07–1.39)	Cao et al. (2020)
RREB1	rs9379084	6086 controls and 2636 GDM	American and Danish	A allele	0.80 (0.71–0.90)	Ding et al. (2018)
SLC30A8	rs3802177	6086 controls and 2636 GDM	American and Danish	G allele	1.17 (1.08–1.26)	Ding et al. (2018)
SHBG	rs6257	359 controls and 359 T2DM	American	C allele	1.68 (1.07–2.64)	Ding et al. (2009)
TCF7L2	rs7903146	810 controls and 210 GDM	German	T allele	1.52 (1.11–2.069)	Fritsche et al. (2018)
		6086 controls and 2636 GDM	American and Danish		1.15 (1.06–1.24)	Ding et al. (2018)
		5639 controls and 1422 T2DM	Swedish	CT + TT vs. CC	1.58 (1.38–1.81)	Lyssenko et al. (2007)
		2501 controls and 150 T2DM	Finish		1.61 (1.14–2.27)	
		6473 controls and 3404 T2DM	Multi-ethnic (Meta-analysis)	TT vs. TC + CC T allele	1.65 (1.42–1.65) 1.53 (1.35–1.72)	Liu et al. (2015)
	rs34872471	6086 controls and 2636 GDM	American and Danish	G allele	1.14 (1.06–1.23)	Ding et al. (2018)
	rs4506565	6086 controls and 2636 GDM	American and Danish	T allele	1.16 (1.08–1.24)	Ding et al. (2018)
	rs12255372	5639 controls and 1422 T2DM	Swedish	GT + TT vs. GG	1.42 (1.24–1.62)	Lyssenko et al. (2007)
TNF-α	rs1800629	181 controls and 196 GDM	Multi-ethnic (Meta-analysis)	A allele	1.38 (0.37–5.16)	Wu et al. (2016)
VEGF	rs2146323	275 controls and 239 GDM	Chinese	AA vs. CC CA + AA vs. CC	2.00 (1.04–3.86) 1.49 (1.05–2.13)	Dong, (2019)

(Continued on following page)

TABLE 1 | (*Continued*) Single nucleotide polymorphisms associated with gestational diabetes mellitus.

Gene	SNP	Number of Participants	Population	Genetic Variant	OR (95%CI)	References
	rs3025039			A allele	1.46 (1.10–1.94)	
				CT vs. CC	1.95 (1.36–2.80)	
				TT vs. CC	6.03 (1.95–18.65)	
				CT + TT vs. CC	2.12 (1.49–3.02)	
				T allele	1.89 (1.42–2.23)	

SNP, single nucleotide polymorphism; OR, odd ratio; CI, confidence interval; GDM, gestational diabetes mellitus; T2DM, type 2 diabetes mellitus.

(OR: 1.58) and a faster deterioration of insulin secretion (Lyssenko et al., 2007; Zhang et al., 2013; Liu et al., 2015). Interestingly, Asian pregnant women homozygous for the TT genotype, had a higher risk of developing GDM (OR = 2.08), followed by Hispanic/Latin (OR = 1.80), and white (OR = 1.51) (Lin et al., 2016). Other ethnic groups have also been studied (Shaat et al., 2007; Zhang et al., 2013; Wu et al., 2016). Therefore, the presence of the T allele in the rs7903146 variant could be a genetic risk factor for GDM. Other SNPs of the same gene have been related with a higher risk to develop GDM in a meta-analysis (Chang et al., 2017), i.e., carriers of the T allele for the rs12255372 variant (OR: 1.46) (Zhang et al., 2013).

TCF7L2 is expressed in several tissues, including the islets of Langerhans, and liver (Zhou et al., 2014). This factor participates as a transcriptional effector in the Wnt signaling pathway where it regulates the transcription of diverse genes, including some of those involved in the production and function of incretin hormones and in blood glucose homoeostasis (Schinner et al., 2008; Ip et al., 2012).

Reduced mRNA levels of this gene in the pancreatic islets are related to a significant increase in the apoptosis of beta cells and a decrease in their proliferation, which causes a reduction in insulin secretion (Shu et al., 2008; Savic et al., 2011). Moreover, evidence in the murine model *Tcf7l2* (null), shown an alteration in glucose metabolism causing hypoglycemia. In contrast, the overexpression of this gene in the same animal model using *Cre* recombinase resulted in glucose intolerance (Savic et al., 2011). This results are related to the high expression levels of *TCF7L2* mRNA in T2DM patients (Lyssenko et al., 2007), and could be an interesting idea for study in GDM models.

The SNP rs7903146 of the *TCF7L2* gene is located in its intronic region upstream exon 5 in a regulatory site, specifically in an islet-selective open chromatin site. This means that in human islet cells, the chromatin state at rs7903146 is more open in chromosomes carrying the T allele, and have a greater enhancer activity compared to the C allele (Gaulton et al., 2010). This could explain the association of the T-allele with an impaired glucose-stimulated insulin secretion (Dahlgren et al., 2007). Moreover, carriers of this risk allele exhibit a significantly higher expression of *TCF7L2* mRNA in the pancreatic islets and an increased hepatic glucose production (Lyssenko et al., 2007). Carriers of the TT genotype for this polymorphism have higher concentrations of blood glucose, proinsulin, and incretin hormones, compared to the normal genotype group (Gjesing et al., 2011). Likewise, the insulinogenic index, derived from an oral glucose tolerance test, is diminished in carriers of the T-allele, which would be associated with an impaired insulin secretion and not with resistance to this hormone (Saxena et al., 2006). However, there are not work in GDM models, or human that demonstrated this association in pregnancies with glucose intolerance.

2.1.2 *HHEX* Genetic Variant Associated to GDM

GWAS have associated several polymorphisms with GDM, and the Hematopoietically-expressed homeobox (*HHEX*) gene rs5015480 stands out among them. This gene codifies for a transcription factor that is part of a homeobox gene family involved in developmental and hematopoietic differentiation processes (Bedford et al., 1993). In a study conducted in Poland, the risk C-allele was associated with a genetic predisposition to develop GDM (OR: 1.40) and also to an increased BMI in pregnant women (Tarnowski et al., 2017). In addition, a meta-analysis showed a strong association of the CC genotype in contrast to the TT genotype (OR: 1.65) in different populations (Wang et al., 2020).

HHEX is a transcription factor that is also linked to the Wnt signaling pathway. It is essential for cell growth and for the development of organs such as the thyroid, pancreas, liver, and brain (Tarnowski et al., 2017). In the adult endocrine pancreas, this factor is selectively expressed in the somatostatin-secreting delta cells, where it has been observed to regulate the differentiation of this cell type (Zhang J. et al., 2014). It is described that a decrease in somatostatin levels causes a paracrine inhibition of the insulin release from β cells (Moldovan et al., 1995). In this context, it has been suggested that this transcription factor directly activates the transcription of the somatostatin gene (Zhang J. et al., 2014).

In the *HHEX* gene, the risk C-allele of the rs5015480 variation is associated with altered β cells secretion. Other variations of this gene are also involved in reduced fasting insulin secretion, insulin sensitivity, and glucose-stimulated insulin secretion (Pivovarova et al., 2009). Some authors have proposed that the rs5015480 variation could affect early stages of insulin secretion due to the reduced insulinogenic index, described in risk allele carriers (Dimas et al., 2014), which could be related to insulin secretion regulation exerted by delta cells (Zhang J. et al., 2014).

Genetic modifications in these transcription factors could cause alterations in the protein expression involved in the development of GDM. In fact, it is proposed that both TCF7L2 and HHEX have an important role in the regulation of insulin secretion in GDM (Saxena et al., 2006; Dimas et al.,

2014). In fact, the polymorphisms *TCF7L2* rs7903146 and *HHEX* rs5015480 could decrease insulin secretion during pregnancy, favoring GDM development due to an alteration at the level of pancreatic cells; and consequently, in the production and secretion of insulin.

2.2 Polymorphisms Associated With Hormones

2.2.1 *ADIPOQ* Genetic Variant Associated to GDM

A relevant hormone in GDM is adiponectin, due to its protective role against insulin resistance. The risk G-allele in rs2241766 (T > G) produces a silent mutation in nucleotide 45 in the adiponectin gene (*ADIPOQ*). This genetic variation is associated with obesity in several studies. Also, carriers of the G/G genotype for this polymorphism had a higher risk of T2DM (OR: 1.70) compared to the T/T genotype (Hara et al., 2002). Moreover, a meta-analysis (Bai et al., 2020) demonstrated an increased risk of GDM in Asian (OR: 2.08) and European (OR: 1.52), but a diminished risk in the American population (OR: 0.642) carriers of the G allele of this SNP.

The adiponectin hormone is synthesized in adipose tissue, where it modulates diverse metabolic processes, among them: metabolism of lipids and fatty acids, reduction of plasmatic triglycerides and improvement of glucose metabolism by an increase in insulin sensitivity (Karbowska and Kochan, 2006). Moreover, adiponectin reduces the expression of adhesion molecules in endothelial cells, the transformation of macrophages into foamy cells, the expression of tumor necrosis factor α (TNF-α) and the proliferation of smooth muscle tissue cells (Mierzyński et al., 2018).

There are genetic variations in the adiponectin gene (*ADIPOQ*) such as rs2241766, where the risk G-allele produces a silent mutation in nucleotide 45, which does not cause an amino acid change. However, being very close to the exon-intron limit, it could affect the splicing machinery (Salazar et al., 2010). The presence of this allele can completely inactivate the activity of the adiponectin promoter and the expression of the *ADIPOQ* gene, and thus can decrease adiponectin levels (Chu et al., 2013).

During pregnancy, the plasmatic levels of this hormone are normally decreased; however, such levels are even lower in the presence of this variation (Huang et al., 2019). In fact, lower levels of adiponectin could be related to an increase in the formation of dense LDL particles (Lara-Castro et al., 2007), and to an increase in the expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 (Hussain et al., 2018).

All this evidence supports the idea that the adiponectin gene could be a susceptibility factor for developing GDM (Takhshid et al., 2015; Xu et al., 2016).

2.2.2 *SHBG* Genetic Variant Associated to GDM

The *SHBG* gene codifies for the sex hormone-binding globulin, which could contribute to the pathophysiology of GDM. This gene has been associated with the risk of diabetes mellitus (Ding et al., 2009; Hedderson et al., 2014). In fact, low levels of pre-gestational SHBG increase the risk of developing GDM (OR: 4.06) (Hedderson et al., 2014).

In addition, the rs6257 (T > C) polymorphism of this gene has been associated with plasmatic concentrations of SHBG. It has been shown that carriers of the CC or CT risk genotypes have lower plasmatic concentrations of this hormone compared to the carriers of the normal TT genotype (Ding et al., 2009; Hedderson et al., 2014). Although there are still not studies that demonstrate association between GDM and these genetics variants, changes in SHBG concentration due to these variants, could increase this risk.

SHBG is synthesized in the liver and is responsible for the transport of androgens in circulation and for the regulation of the bioavailability of these hormones. It is also involved in receptor-mediated processes where it regulates the effects of dihydrotestosterone (DHT) and estradiol (Fortunati, 1999).

An *in-silico* study postulates that rs6257 favors the union of the *forkhead box protein A2* (FOXA2) element to the *SHBG* gene, repressing its transcription by a splicing defect (Ding et al., 2009). This idea was tested in HepG2 cells (Wu, 2015). Furthermore, it is described that in GDM there is a decrease in the plasmatic concentrations of SHBG compared to pregnant women without this pathology (Bartha et al., 2000; Tawfeek et al., 2017; Faal et al., 2019). This phenomenon was also studied in a trophoblast cell model (HTR8 Sv-neo) exposed to high levels of insulin, showing a decrease in SHBG mRNA and protein levels (Zhang et al., 2016; Feng et al., 2018). This could be explained by a reduction in the signaling of the phosphatidylinositol 3-kinase (PI3K/Akt) pathway, which mediates the transduction of insulin signals (Feng et al., 2018). Although literature is scarce, the reduction of GLUT-4, GLUT-3 and IRS-1 expression in GDM patients could be correlated with a lower SHBG activity, which could favor insulin resistance (Zhang et al., 2016).

2.3 Polymorphisms Associated With Membrane Proteins

2.3.1 *GNB3* Genetic Variant Associated to GDM

Heterotrimeric G-proteins are relevant components of transmembrane receptors and are involved in the regulation of different intracellular signaling pathways. The 825C > T SNP in the gene of the G-protein β3 subunit (*GNB3*) has been linked to metabolic features such as hypertension, atherosclerosis and immunological response. The T-allele has also been associated with obesity risk in German, Chinese and South African populations (Siffert et al., 1999). In pregnant women, carriers of the risk allele have a higher weight gain during gestation (Dishy et al., 2003). Interestingly, it has also been described that the CT and TT genotypes are significantly related to a higher risk of GDM (OR: 1.91; 95%CI: 1.053–3.463) (Feng et al., 2019); however, there is still a lack of studies to confirm this relationship.

The G protein is involved in the regulation of glucose levels through the metabolic pathway of insulin signaling. Also, it is involved in the stimulation of second messengers such as adenylate cyclase, epinephrine signaling pathways and glucagon receptors in the liver, muscular and fatty tissue cells (Rizvi et al., 2016).

The most studied polymorphism of the *GNB3* gene is C825T, which is produced in exon 10 and it generates an alternative splicing causing the loss of 41 amino acids, structurally modifying this protein. The risk T-allele has been associated with a higher production of the G protein beta three subunit, causing an increased activation of this protein. With that increased activation comes an enhanced activity of the potassium channels at the cardiac level and vasoconstriction mediated by α-adrenoreceptors, which are directly related with arterial hypertension (Andersen et al., 2006). Another study demonstrated that the CC genotype of this SNP might be associated with higher obesity-related metabolic traits, such as triglyceride and total cholesterol in non-obese subjects (Hsiao et al., 2013).

Unfortunately, there is no direct mechanism that explains the relationship of this polymorphism and insulin resistance. However, obesity-related metabolic traits are tightly linked to GDM pathophysiology.

2.3.2 *KCNQ1* Genetic Variant Associated to GDM

It is suggested that the gene of the potassium voltage-gated channel subfamily Q member 1 (*KCNQ1*), participates on the regulation of insulin secretion in the pancreas, and it has been described as one of the candidate genes for GDM. Evidence shows that the rs2237892 variation of this gene is significantly associated with increased glucose levels, impaired insulin secretion, and higher GDM risk (OR: 1.99) in Asian population (Yasuda et al., 2008; Shin et al., 2010; Ao et al., 2015). Correspondingly, a study including 637 Pakistani women associated the presence of the risk allele A of this polymorphism, with an enhanced GDM risk (OR: 2.07) (Fatima et al., 2016). On the other hand, the risk genotype of rs163182 has been associated with lower GDM risk (OR: 0.84) (Cao et al., 2020).

The *KCNQ1* gene encodes for the alpha subunit of the pore-forming potassium channel (KvLQT1) performing an important role in the control of the vascular repolarization process (Yasuda et al., 2008). This gene is expressed in epithelial cells, including those of the endocrine and exocrine pancreas. In addition, KCNQ1 channels are expressed in insulin-secretor INS-1 cells, where they depolarize the membrane potential of the pancreatic beta cells allowing insulin secretion (Kwak et al., 2010).

Genetic variations in the sequence of *KCNQ1* such as rs2237892 cause changes in the translation and/or in post-translational modifications, reducing β cells function (Jonsson et al., 2009). Thus, a decreased secretory capacity of the β cells could increase GDM risk by reducing the secretory capacity of insulin and limiting its compensation (Wang et al., 2013). In fact, GDM patients carrying the risk allele have higher fasting plasma glucose levels and lower insulin secretion (Wang et al., 2013).

2.4 Polymorphisms Associated to Enzymes

2.4.1 *DIO2* Genetic Variant Associated to GDM

The *DIO2* gene codifies to an enzyme that is ubiquitous in the human body, and that regulates the conversion of tetraiodothyronine (T4) to triiodothyronine (T3). It is proposed that the rs225014 (T > C) polymorphism causes a change in the amino acid sequence from a threonine (Thr) to an alanine (Ala) on codon 92 (Thr92Ala) (Estivalet et al., 2011). This variation has been associated with higher GDM risk (OR: 1.29) (Asadi et al., 2016).

Deiodinase 2 (DIO2) beside the essential role in thyroid hormones homeostasis, is also involved in brain growth and maturation, glucose uptake in the muscle, among other effects (Loubiere et al., 2010; Galton et al., 2014; Yang et al., 2016). This enzyme is mainly found in the central nervous system, hypophysis, skeletal muscle, thyroid, heart, bones, and adipose tissue (Coppotelli et al., 2006).

The rs225014 genotype is associated with *DIO2* expression (Bomer et al., 2015), and the presence of the risk C allele, which causes the substitution of a Threonine for an Alanine, reducing the DIO2 activity (Estivalet et al., 2011). In fact, it has been observed that homozygous patients for this polymorphism have a lower enzymatic activity, evidenced by a 37% decrease in DIO2 speed in skeletal muscle, and a reduction around 90% in its maximal velocity (V_{max}). These results may explain the association of this variation with insulin resistance. As the skeletal muscle is the main site of insulin-dependent glucose uptake, a lower DIO2 activity would decrease the amount of T3 generated in the skeletal muscle, and with this, the expression of genes involved in energy use, such as GLUT4, leading to insulin resistance (Canani et al., 2005).

2.4.2 *FTO* Genetic Variant Associated to GDM

The fat mass and obesity-associated gene (*FTO*) codifies for a dioxygenase enzyme that is found within the cell nucleus. It is reported that *FTO* participates in mRNA processing and splicing processes. Regarding the *FTO* gene, the polymorphisms rs9939609, rs8050136, and rs1421085 are associated with BMI increase, risk of obesity, and T2DM. However, controversy still exists about the association between certain polymorphisms and GDM (Hotta et al., 2008; He et al., 2018). Indeed, rs8050136 and rs14211085 SNPs were not associated with GDM risk (de Melo et al., 2015; Anghebem-Oliveira et al., 2017; Lin et al., 2018; Tarnowski et al., 2018), but with proinflammatory state and weight gain during pregnancy (Saucedo et al., 2017).

The FTO rs9939609 (T > A) polymorphism is located in the first intron of this gene (Frayling et al., 2007). Recent evidence suggests that this polymorphism is strongly correlated with GDM risk (OR: 1.31) in Caucasian subjects (Lin et al., 2018). Also, the A-allele is associated with rapid weight gain during pregnancy (Lawlor et al., 2011; Martins et al., 2016).

These genetic variants could alter the expression or enzymatic activity of FTO, leading to changes in the metabolism that could impair glucose metabolism and generate insulin resistance (Saber-Ayad et al., 2019). Subsequently, the GDM risk could be increased.

The FTO protein acts within the nucleus demethylating the N6-methyladenosines in mRNA and regulating the splicing of genes involved in adipogenesis such as *FABP4*, *PPARγ*, *C/EBPα* and *PLIN1* (Zhao et al., 2014a; Ben-Haim et al., 2015; Merkestein et al., 2015; Zhang et al., 2015; Bartosovic et al., 2017).

In murine models, the *Fto* gene is widely expressed in the brain, including the hypothalamic nucleus, which is related to energy intake regulation (Speakman, 2015). Studies in primary culture of Mouse Embryonic Fibroblasts (MEFs) have demonstrated that FTO regulates the splicing of the *RUNX1T1* (Runt-related transcription factor 1) gene (Zhao et al., 2014b), which is involved in the early stages of adipogenesis (Rochford et al., 2004). FTO exerts its action

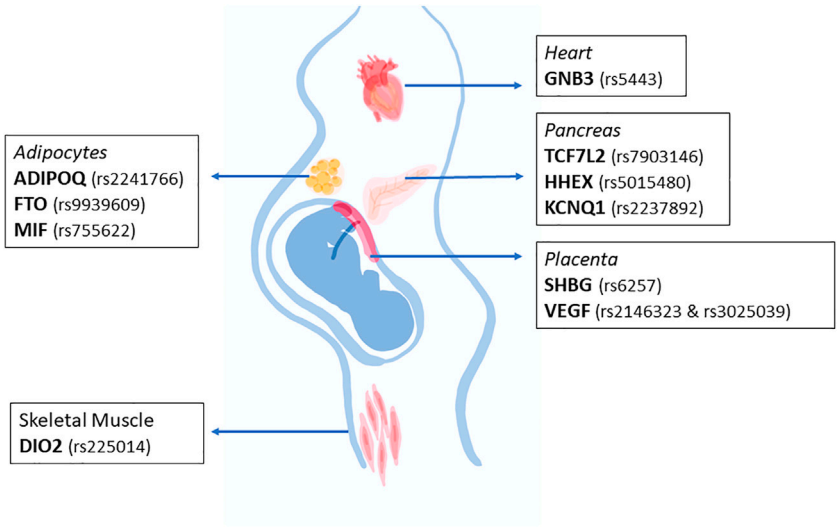

FIGURE 1 | Major organs affected by the main SNPs associated with gestational diabetes mellitus. The main organs affected by the SNPs described in this review are heart, adipose tissue, pancreas, and skeletal muscle. Interestingly, the SNPs described would affect some functions in the indicated organ, favoring the onset of gestational diabetes mellitus.

during the splicing of the mRNA transcript of this gene, causing the exclusion of exon 6 and generating a short pro-adipogenic isoform of RUNX1T1, which increases adipocytes proliferation. The latter is a condition that is favored when *FTO* is overexpressed (Merkestein et al., 2015). Moreover, *FTO* overexpression in C57B/6J mice is associated with weight gain and increased adipogenic activity (Merkestein et al., 2015).

Variations in the first intron of the FTO gene have been associated with higher BMI and T2DM, and it has been reported that there is a 47 kb region that comprehends several SNPs associated with these pathologies. The rs9939609 variant has been extensively studied (Frayling et al., 2007). The mechanism by which this variant causes obesity is still unclear, however, heterozygous subjects for this polymorphism have higher levels of primary FTO transcripts of the risk A-allele, than of the T-allele (Berulava and Horsthemke, 2010), and this could cause higher levels of FTO expression favoring adipogenesis. However, the latter relationship has not yet been reported in literature.

Another SNP close to rs9939609 and associated with an increased BMI is rs8050136. The DNA sequence that included A-allele of this polymorphism preferentially binds with CUTL1, a transcription factor that increases the FTO expression. It has been proposed that, given the proximity of these variants, the rs9939609 SNP could also increase FTO expression by the same mechanism (Stratigopoulos et al., 2008).

2.5 Polymorphisms Associated With Growth Factors

2.5.1 *VEGFA* Genetic Variant Associated to GDM

Another group of genes associated with GDM are growth factors. One of them is the vascular endothelial growth factor A (*VEGFA*), which has an essential role in angiogenesis by inducing the migration of endothelial precursor cells from the bone marrow, and causing differentiation and proliferation of endothelial progenitor cells in angiogenesis sites (Saavedra et al., 2017). A study involving the *VEGFA* gene showed that the A allele of the rs2146323 polymorphism and the T allele of the rs3025039 variant, increase the risk of developing GDM (OR: 1.456 and 1.894, respectively) (Dong, 2019). Additionally, the frequency of the risk haplotypes of rs2010963, rs833069, rs2146323 and rs3025010 is higher in GDM patients compared to normal pregnancies (Dong, 2019).

VEGFA has the function of promoting endothelial cell proliferation and increasing vascular permeability to induce angiogenesis. In fact, the placentas of GDM patients show hypervascularization, which is explained by a greater demand of oxygen by the fetus due to an increase in fetal aerobic metabolism stimulated by insulin (Troncoso et al., 2017). A study conducted in a murine model with GDM induced by a high fat diet, showed that those who had GDM had a high placental inflammatory response evidenced by increased IL-1β and TNFα, and a higher degree of placental hypoxia denoted by an enhanced expression of inducible hypoxia factor-1α (HIF-1α) and VEGF-A. In this sample, altered placental vascular development due to hypervascularization was also present (Li, 2013). Therefore, in GDM, VEGF would be involved in angiogenesis processes in the placenta, but it is not clear whether genetic alterations could explain this mechanism. In fact, variations of the VEGF gene (rs2146323 and rs3025039) have only been associated with an increased risk of GDM, and therefore could be caused by an abnormal expression of VEGF, increasing its levels in these patients (Dong, 2019).

However, molecular mechanisms explaining this dysfunction have not been described for these polymorphisms. Evidence for rs735286 indicates that it is located in the second VEGF intron, and this variant involves a region that has putative binding sites

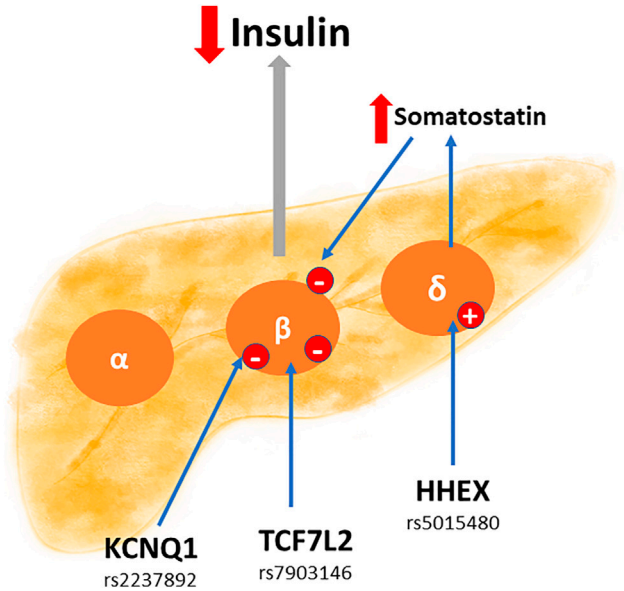

FIGURE 2 | Potential SNPs capable of reducing the production of maternal insulin at a pancreatic level. Some SNPs can affect the secreting function of pancreatic insulin. In this sense, the SNPs *KCNQ1* rs2237892, *TCF7L2* rs7903146 and *HHEX* rs5015480, reduce (down red arrow) direct or indirectly the production and secretion of insulin at the level of the beta cells (β). Moreover, the same *HHEX* SNP can stimulate (up red arrow) somatostatin secretion in delta cells (δ), and this hormone is an inhibitor of insulin secretion. Impaired insulin production favors insulin resistance, and therefore the appearance of gestational diabetes mellitus.

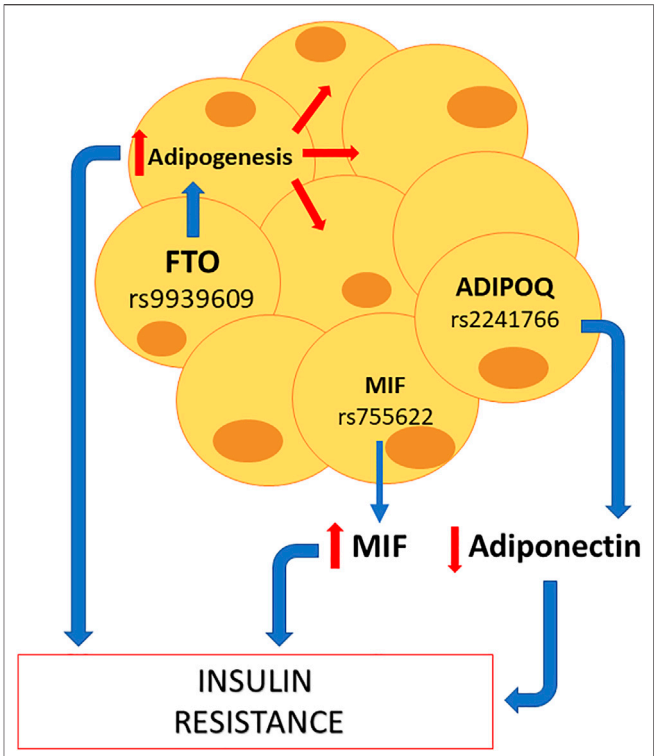

FIGURE 3 | Potential SNPs capable of promoting maternal insulin resistance at adipose level. Some SNPs that can affect the adipocyte function are *FTO* rs9939609, which increases adipogenesis; *MIF* rs755622, which increases MIF levels; and *ADIPOQ* rs2241766, which reduces adiponectin levels. All these three effects favor the appearance of insulin resistance, and subsequently, gestational diabetes mellitus.

for transcription factors such as the myeloid zinc finger protein (MZF-1), that regulates *VEGFA* expression. Therefore, rs735286 could affect the transcription process and cause changes in splicing (Morris et al., 1994; Churchill et al., 2008).

Although the rs2146323 polymorphism is also found in intron 2, it is located relatively distant from these binding sites, so the mechanism would be apparently different (Churchill et al., 2008). On the other hand, it has been suggested that rs3025039, being in a 3′-UTR region, could alter the conformation of the mRNA, which would decrease the transcript degradation, causing an increase in VEGF expression (Dibbens et al., 1999; Tahara et al., 2009).

2.5.2 *MIF* Genetic Variant Associated to GDM

The macrophage migration inhibitory factor (MIF), is a pro-inflammatory cytokine secreted by T-lymphocytes in response to delayed-type hypersensitivity, which exerts an inhibitory effect on macrophage migration. The rs755622 polymorphism of this gene (MIF-173G/C) is associated with higher GDM risk (OR: 1.59) (Li et al., 2016), and the rs1007888 polymorphism is related to high levels of blood glucose and insulin (Zhan et al., 2015).

MIF is a pro-inflammatory cytokine that is secreted in response to delayed-type hypersensitivity and exerts an inhibitory effect on macrophage migration (Hamidi et al., 2019). It has been described that MIF controls underlying metabolic and inflammatory processes during periods of stress, regulating glucose homeostasis and macrophage infiltration into adipose tissue. Also, MIF is expressed and secreted by adipose tissue, evidencing higher levels in obesity (Mejia-Montilla et al., 2015).

The rs755622 variation is located in the promoter region of *MIF* and the C allele is related with a higher transcriptional activity of the *MIF* gene. In fact, patients with T2DM have shown increased MIF levels (Hamidi et al., 2019). Also, it has a direct association with insulin resistance through the production of some pro-inflammatory cytokines and adipokines, including resistin and IL-6. Additionally, this variation has been related to an increased risk of GDM in Chinese women; hence, more studies should be carried out in other populations to determine the role of this polymorphism in this pregnancy disease (Li et al., 2016). However, more studies are needed to confirm the association of these variants and GDM.

2.6 Other SNPs Associated With GDM

The IRS-1 is an intracellular adaptor protein that plays a key role in insulin signaling. The *IRS1* rs1801278 (C > T) variant has been related to GDM in meta-analyses (Zhang Y. et al., 2014; Wu et al., 2016), similarly to the rs7578326 (A > G) polymorphism (Voight et al., 2010; Zheng et al., 2013; Zhao et al., 2017). *IRS1* rs1801278 is a missense polymorphism that decreases the phosphorylation of IRS-1 *in vitro* (McGettrick et al., 2005), reducing the binding of p85 to IRS-1 and the activity of PI3K in different cell lines (Hribal

et al., 2000; Sentinelli et al., 2006), and leading to insulin resistance. On the other hand, rs7578326 is an intron variant positioned in a distal regulatory element that targets *IRS1* (Lu et al., 2013). The risk allele A removes a CpG site and avoids its methylation (Dayeh et al., 2013). This genetic variant has been linked to higher levels of transcript in skeletal muscle, with no changes in the levels of IRS1 mRNA (Soyal et al., 2015). Further studies are needed to elucidate the exact role of this SNP on the pathophysiology of T2DM and GDM.

The SNP 43, SNP 44, SNP 63, and Indel 19 variants of the *CAPN10* gene were not associated with GDM risk (Shaat et al., 2005; Neuhaus et al., 2013; Khan et al., 2014; Zhang et al., 2019; Ustianowski et al., 2021), except in some genetic association models of SNP 63 and SNP 44 (Cui et al., 2016), and in *CAPN10* SNP-43/19/63 haplotypes (Hou et al., 2017). Nevertheless, the Indel-19 and SNP-19 variants were associated with higher glucose levels in GDM women (Castro-Martínez et al., 2018). In addition, GWAS have discovered several genetic variants associated with both T2DM and GDM. Among them, the rs7754840 polymorphism of the *CDKAL1* gene (C-allele) and the rs10830962 polymorphism of the *MTNR1B* gene (G-allele) have been associated to a higher GDM risk (OR: 1.518; OR: 1.454, respectively). The risk alleles of these polymorphisms have also been associated with a decreased fasting insulinemia in GDM women (Kwak et al., 2012).

In a meta-analysis, other polymorphisms were associated with the risk of developing GDM, such as the A allele of the rs1800629 polymorphism in the TNF-α gene (OR 2.69), the T-allele of the rs4402960 SNP in the *IGF2BP2* gene (OR: 1.22), and the G-allele of the rs10830963 variant in the *MTNR1B* gene (OR: 1.28). This last variant is also associated with GDM risk, in Asian (OR 1.23) and Caucasian (OR: 1.49) populations, and women with pre-gestational BMI ≥25 kg/m^2 (OR 1.24) (Wu et al., 2016).

3 CONCLUDING REMARKS

Scientific literature has evidenced various polymorphisms associated with GDM, which can affect one or several functions. However, the specific mechanism by how these polymorphisms can affect the body physiology has not been addressed. **Figure 1** summarizes the main tissues that would be affected by the described polymorphisms during pregnancy. We emphasize that the main organs associated with energy metabolism such as skeletal muscle, adipose tissue and pancreas could be involved, but clearly the placenta is an organ that plays an important role in the context of a pregnancy-related pathology. Although the placenta is a fetal organ that is genetically different from the mother, it is difficult to know whether maternal genetic alterations could be linked to placental defects.

The endocrine pancreas is a key organ in the synthesis of several hormones such as somatostatin, glucagon, and insulin. The latter is relevant to understand the pathophysiology of GDM. In fact, as mentioned earlier, GDM courses with supraphysiological insulin resistance. Accordingly, and as shown in **Figure 2**, the polymorphisms *KCNQ1* rs2237892, *TCF7L2* rs7903146, and *HHEX* rs5015480 are closely linked in the reduction of insulin secretion by beta cells, which could explain GDM development. One cause of insulin resistance is obesity. That explains why polymorphisms that have a consequence on metabolism, such as adipose tissue hyperplasia, are associated with GDM, as shown in **Figure 3**. Indeed, the polymorphisms *FTO* rs9939609, *ADIPOQ* rs2241766, and *MIF* rs755622 are strongly linked to the presence of insulin resistance.

Unfortunately, evidence is insufficient to understand all the pathophysiological changes observed in GDM at the genetic level. In fact, GDM, as well as T2DM, are polygenic pathologies. However, current scientific evidence leads us to believe that certain polymorphisms could favor alterations in key organs, such as the pancreas and the adipose tissue, promoting insulin resistance during pregnancy and GDM.

AUTHOR CONTRIBUTIONS

BO-C and AA tabulated literature information; BO-C, AA, JAP, MG, EC, AO, JG, LL and JAR prepared the figures and improved the manuscript; BO-C, DM and EG-G wrote the manuscript.

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