ENDOCRINE AND METABOLIC DISEASES – GENETIC IMPACT AND THERAPIES

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ENDOCRINE AND METABOLIC DISEASES – GENETIC IMPACT AND THERAPIES

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Editorial: Endocrine and metabolic diseases – genetic impact and therapies

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Editorial on the Research Topic
Endocrine and metabolic diseases – genetic impact and therapies

Genetics plays an important role in endocrine and metabolic diseases. Despite many advances on genes implicated in these disorders and associated signaling pathways, there remain major challenges: an improved understanding of the heterogeneity of complex endocrine and metabolic disorders, especially obesity and diabetes, a better assessment of the risk associated with susceptibility genetic factors, and to improve personalized medicine. This Research Topic of *Frontiers in Endocrinology* explores through a number of complementary examples the latest work and emerging ideas related to the identification of genetic factors involved in endocrine and metabolic diseases, novel signaling pathways, translational research, and clinical applications.

The genetic contribution to diseases is usually evaluated by heritability, which is an estimate of how much of the disease susceptibility is attributable to genetic variations. The search for contributing genes in monogenic disorders started in the 1990's, using large familial pedigrees and linkage analysis followed by candidate gene approaches. Many important discoveries have been made by applying this strategy in all fields of Mendelian genetics. Pathogenic variants, which segregate in families were found to be associated with highly penetrant disorders, thereby providing the first insights into the pathophysiology of the corresponding conditions. A second era in the identification of monogenic disorders occurred with the advent of exome sequencing, which revolutionized our understanding of rare diseases, uncovering causal rare variants for hundreds of these disorders. Besides disorders of Mendelian inheritance, the search for

genetic variants that contribute to common multifactorial forms of endocrine and metabolic diseases started later, in the 2000's, with the development of genome-wide association studies (GWAS). GWAS led to the identification of thousands of genetic loci associated with complex diseases. Nevertheless, identifying the causal gene(s) and/or variant(s) within each association locus remains an ongoing challenge. Increasing availability of high-throughput genome-scale technologies, advanced computational tools, comprehensive multi-omics databases should accelerate the translation of GWAS loci into meaningful biological markers. In certain conditions, such as diabetes or liver steatosis, polygenic scores have been proposed. A polygenic score represents an individual's genetic susceptibility to develop a disease and is calculated by summing the number of susceptibility genetic variants, weighted by each variant's effect size observed in a GWAS. Based on such genetic profiling, several direct-to-consumer genomic companies are already informing individuals about their risk and predisposition for a panel of common diseases and traits, including obesity and type 2 diabetes. Although these results have fueled expectations that genotype information could be used in clinical care for early diagnosis of high-risk individuals, the current quality of these predictions remains quite low (1). In between Mendelian genetics and GWAS, now appears the possibility to use data from UK Biobank, which contains detailed phenotypic data linked to medical records for approximately 500,000 participants, offering an unprecedented opportunity to evaluate the effect of rare variations on a broad collection of traits (2).

In addition to the genetic risk, the appearance and evolution of endocrine and metabolic disorders can be influenced by a number of environmental factors including socio-demographic, lifestyle, and clinical characteristics. Genes and environmental factors can also interact to modify our metabolism. Epigenetics, which corresponds to biochemical modifications influencing gene expression and activity, can regulate cellular processes and the whole-body physiology, as observed in type 2 diabetes (3). Another environmental factor that impacts the pathophysiology of endocrine disorders is the control of circadian rhythms (4). Endocrine organs release a variety of hormones in response to diurnal cycles of light/dark, fasting/feeding, and temperature changes (5). This is of clinical relevance since disruption of the circadian clock is linked to metabolic disease. The inflammatory state of a patient is also a determinant of the risk of type 2 diabetes and obesity. This is illustrated by the rapid development of a new field called "immunometabolism," which studies the complex interactions between metabolic and inflammatory pathways in immune and metabolic tissues (5).

Personalized medicine is defined as the right treatment for the right person at the right time. To date, there are few examples of precision therapeutics. Patients with congenital leptin deficiency can benefit from leptin replacement to treat severe obesity (6). Patients with monogenic diabetes due to pathogenic variants in the genes encoding the KCNJ11 and ABCC8 potassium channel subunits are very responsive to sulfonylureas and do not need insulin therapy (7). Patients with monogenic diabetes due to *GCK* variants display a mild form of diabetes, stable over time, which does not require any treatment. The ambition to personalize all aspects of an individual's management, including precision diagnosis, lifestyle and treatment, and prognosis has recently been underlined by international consortia in the case of diabetes (7). More comprehensive approaches comprising demographic, environmental, genetic, clinical, and biological markers will be needed to accurately predict who is at risk of a given condition. Classification in subtypes of heterogenous diseases, like obesity and type 2 diabetes, will also be a major challenge, and delineation of the diverse underlying biological mechanisms will be a pre-requisite to tailor prevention and treatment strategies.

Author contributions

IJ, BF, RJ: wrote and validated the last version of the Editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

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

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SYSTEMATIC REVIEW
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ABCA1 69C>T Polymorphism and the Risk of Type 2 Diabetes Mellitus: A Systematic Review and Updated Meta-Analysis

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Background: The ATP-binding cassette transporter A1 *(ABCA1)* is likely associated with the risk of type 2 diabetes mellitus (T2DM) *via* β cell function modification, but the evidence on the association remains unclear. This study aimed to investigate the relationship between the *ABCA1* 69C>T polymorphism and the risk of T2DM through a systematic review and meta-analysis.

Materials and Methods: The PubMed, Web of Science, and Embase databases were searched for qualified studies published until August 2020. Studies that included the association between the *ABCA1* 69C>T polymorphism and the risk of T2DM were reviewed. The odds ratios (ORs) and 95% confidence intervals (CIs) were evaluated.

Results: We analyzed data from a total of 10 studies involving 17,742 patients. We found that the CC or CT genotype was associated with increased risk of T2DM than the TT genotype (OR, 1.41; 95% CI, 1.02-1.93). In the Asian population, the C allele carriers had a higher risk of T2DM than those with the TT genotype; the ORs of the CC and CT genotypes were 1.80 (95% CI, 1.21-2.68) and 1.61 (95% CI, and 1.29-2.01), respectively.

Conclusions: This meta-analysis confirmed that the *ABCA1* 69C>T genotype showed a decrease risk of T2DM compared to the CC or CT genotypes.

Keywords: *ABCA1* 69 C>T, meta-analysis, systematic review, polymorphism, type 2 diabetes mellitus

INTRODUCTION

Diabetes is a major global health issue estimated to have affected approximately 463 million people in 2019, with this number predicted to reach 700 million by 2045 according to the International Diabetes Federation (1). In addition, the annual cost of diabetes care is USD 760 billion (2). Diabetes is a serious, chronic endocrine disease that occurs when the blood glucose level is elevated due to insufficient insulin secretion and low sensitivity of target organs or cells to insulin (3–5). Diabetes is also associated with several comorbidities such as neuropathy and angiopathy, which have become leading causes of mortality and morbidity worldwide (1, 6).

Diabetes is classified into 2 types, and type 2 diabetes mellitus (T2DM) is the most prevalent. The development of T2DM is complex and involves a combination of several genetic and environmental factors (7–9). Several genes contribute to the overall susceptibility to T2DM by influencing the baseline glucose tolerance level (10). Genetic linkage analysis and association studies have identified several candidate genes contributing to T2DM.

The ATP-binding cassette transporter A1 (*ABCA1*) is considered an important gene that can modify β cell function, although its primary function is associated with cholesterol metabolism. ABCA1 contributes to the reverse transportation of cholesterol from peripheral tissues to the liver *via* high-density lipoprotein-cholesterol (HDL-C). Polymorphism in the *ABCA1* gene is reportedly related to HDL-C deficiency, which leads to coronary heart disease or coronary artery disease (11–13). *ABCA1* may also be crucial to maintaining β cell cholesterol homeostasis and function (14, 15). Because cholesterol is an important factor for membrane organization and survival of β cells, cholesterol accumulation in β cells impairs glucose metabolism and reduces insulin secretion, resulting in the development of T2DM (16). A study showed that β cell–specific *Abca1* knockout mice had significantly higher fasting blood glucose levels than their littermate controls because of a defect in the first-phase glucose-stimulated insulin release (17). Studies have shown that several common variants of *ABCA1* gene are also associated with the development of T2DM in humans (18). However, evidence regarding the association between *ABCA1* gene polymorphisms and the risk of T2DM remains unclear. Therefore, we aimed to investigate the relationship between the extensively studied *ABCA1* 69C>T polymorphism and the risk of T2DM through a systematic review and meta-analysis.

METHODS AND MATERIALS

Literature Search Strategy

Two researchers independently searched three databases (PubMed, Web of Science, and Embase) in August 2020 for studies on the association between *ABCA1* 69C>T and T2DM. The following search terms were used: [(ABCA1 OR ATP-binding cassette transporter 1 OR ATP-binding cassette transporter A1 OR adenosine triphosphate-binding cassette transporter A1 OR ATP Binding Cassette Sub Family A Member 1 OR ATP Binding Cassette Transporter, Subfamily A) AND (polymorph* OR variant* OR mutation* OR genotyp* OR allele* OR SNP*) AND (diabetes mellitus OR diabet* OR NIDDM OR T2D* OR T2DM)]. The search was not restricted by publication date. Duplicates and irrelevant studies were removed through the initial screening of titles and abstracts according to the eligibility criteria. This meta-analysis was conducted according to the checklist outlined in the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (19).

Study Inclusion and Exclusion Criteria

The following criteria were used to identify eligible studies: (i) evaluating the association between the *ABCA1* polymorphisms and the risk of T2DM; (ii) using prospective or retrospective cohort study or case-control study design; (iii) providing sufficient information to calculate odds ratios (ORs) and 95% confidence intervals (CIs); and (iv) being published in English. Exclusion criteria included: (i) conference or meeting abstracts, summaries, reviews, comments, letters, news, and editorials; (ii) *in vitro* or animal studies; or (iii) unable to extract the data. In case of overlapping data, only the most recent and comprehensive data were included in the meta-analysis.

Data Extraction and Quality Assessment

All data were extracted independently by two researchers, and discrepancies were resolved by consensus. The following information was extracted from each study: name of the first author, publication year, study design, country, the number of participants, percentage of T2DM and females, age, body mass index (BMI), genotyping method, and the Newcastle–Ottawa scale (NOS) score. Two researchers independently assessed the selected studies based on the NOS for cohort studies and case-control studies (20). NOS has three categories: selection of study sample, comparability between the case and control groups, and outcome or exposure assessment. Each study can be assessed with a total score of 0-9. In this review, we rated 1 point for each item of comparability, if age and other known risk factors (such as BMI) were matched or adjusted for in the analysis.

Statistical Analysis

Review Manager (version 5.3; The Cochrane Collaboration, Copenhagen, Denmark) was used for data review. ORs and 95% CIs were calculated using the Z test to estimate the strength of the association between the *ABCA1* 69C>T polymorphism and the risk of T2DM. A p value <0.05 was considered statistically significant.

The heterogeneity across studies was estimated using a chi-square test, and an I^2 statistic. I^2 >50% was considered to indicate significant heterogeneity. In the absence of any statistical evidence of heterogeneity, the fixed-effects model was used; otherwise, the random-effects model was used to calculate pooled estimates (21, 22). Subgroup analysis was performed according to ethnic groups. Both the Begg test and the Egger regression test of the funnel plot were conducted using R Studio software (version 3.6.0; R Foundation for Statistical Computing, Vienna, Austria) to identify publication bias (23, 24).

RESULTS

Literature Search and Characteristics of Included Studies

A detailed flow chart of the study selection process is presented in **Figure 1**. A total of 571 studies were retrieved through the electronic databases. After duplicate removal, 360 records were initially identified, and the titles and abstracts were screened for inclusion in the study. From this initial review, the full texts of 26 studies were assessed for eligibility. Of these studies, 17 were excluded for the following reasons: not original articles (n = 4), not having appropriate outcomes (n = 4), and not containing *ABCA1* 69C>T outcomes (n = 9). One study was added through manual search. Thus, 10 articles were identified for this meta-analysis. All the 10 articles were written in English.

The characteristics of the studies included are summarized in **Table 1**. The studies were published between 2012 and 2020, most of them were case-control studies, and they mainly included Asian populations. Quality scores evaluated using the NOS for all included studies ranged from 4 to 8 (**Table 1**).

Associations of the *ABCA1* 69C>T With T2DM

Ten studies with a total of 17,742 participants were evaluated for the association between *ABCA1* variants and the risk of T2DM (18, 25–33) (**Figure 2**). Because significant heterogeneity by the chi-square and I^2 tests ($I^2 > 50\%$) was found, the analysis was conducted using the random-effects model to calculate the pooled ORs. The CC or CT genotype was associated with increased risk of T2DM than the TT genotype (OR, 1.41; 95% CI, 1.02-1.93; **Figure 2A**). Neither the Begg test nor the Egger test showed significant publication bias (Begg test, p = 0.655; Egger test, p = 0.958, **Supplementary Figure 1**). Sensitivity analysis was performed by sequentially excluding each study; the estimates showed a similar trend, with ORs ranging from 1.31 to 1.56.

In respective comparisons of the three genotypes, the CC genotype was associated with 1.1-fold (95% CI, 0.91-1.37) and 1.5-fold (95% CI 1.02-2.15) higher risk of T2DM compared to the CT and TT genotypes, respectively (**Figures 2B, C**), although statistical significance was only obtained in the comparison between the CC and TT genotypes. Regarding the CT and TT genotypes, the CT genotype had 1.4-fold (95% CI, 1.03-1.80) higher risk of T2DM compared to the TT genotype (**Figure 2D**).

Subgroup Analysis in Asians

In the subgroup analysis for the Asian population, we found that the C allele carriers had significantly higher risk of T2DM than those with the TT genotype (OR 1.68; 95% CI, 1.24-2.28; **Figure 3A**). Although the CC genotype was associated with no significant risk of T2DM compared to the CT genotype among Asians (OR, 1.13; 95% CI, 0.87-1.48; **Figure 3B**), the CC and CT genotypes were associated with 1.8-fold (95% CI, 1.21-2.68; **Figure 3C**) and 1.6-fold (95% CI, 1.29-2.01; **Figure 3D**) higher risks than the TT genotype, respectively.

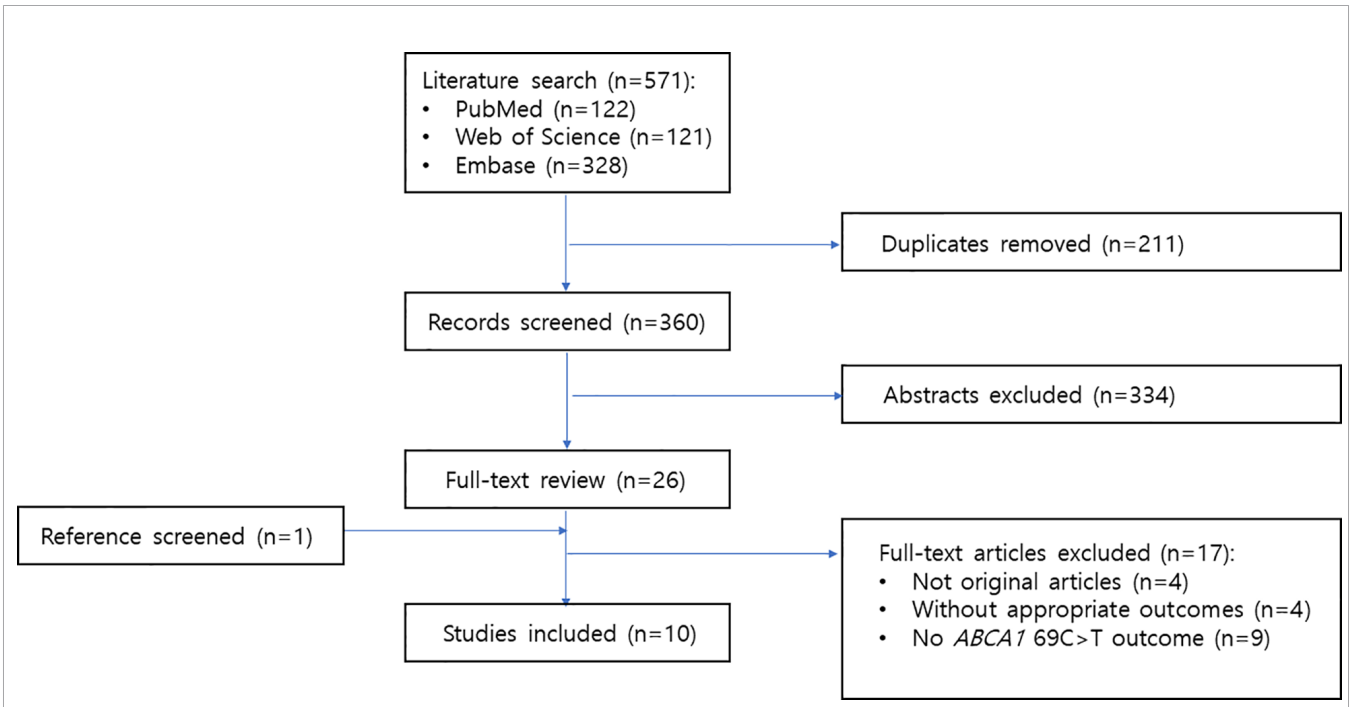

FIGURE 1 | Flow diagram of included studies.

TABLE 1 | Characteristics of studies included in the systematic review.

First author, year, ref number	Study design	Country	Participants		Age (years) T2DM/ Control(mean ± SD)	BMIT2DM/Control (kg/m^2± SD)	Genotyping	NOS
			Total (T2DM %)	Female % T2DM/Control				
Alharbi, 2013 (25)	Case-control study	Saudi Arabia	756 (49.7)	40.2/46.8	50.6 ± 10.4/46.0 ± 7.7	29.5 ± 5.9/29.2 ± 5.5	PCR-RFLP	6
Du, 2020 (26)	Case-control study	China	1998 (49.9)	50.2/49.6	60.2 ± 8.6/59.7 ± 8.9	26.4 ± 3.2/25.1 ± 3.6	SNaPshot	7
Ergen, 2012 (27)	Case-control study	Turkey	157 (68.2)	66.4/34.0	56 (25-85)/49 (29-85)[1]	27.5 ± 5.0/25.2 ± 3.8	PCR-RFLP	6
Ghafar, 2020 (28)	Case-control study	Egypt	197 (52.8)	62.5/51.6	49.7 ± 9.0/48.0 ± 9.7	29.2 ± 4.1/22.6 ± 2.9	TaqMan real-time PCR	7
Haghvirdizadeh, 2015 (29)	Case-control study	Malaysia	329 (49.9)	37.2/47.3	62.1 ± 9.6/55.0 ± 11.8	27.9 ± 5.1/27.1 ± 6.2	PCR-HRM	5
Hasan, 2019 (30)	Case-control study	Bangladeshi	200 (51.0)	70.6/69.4	40.4 ± 1.3/39.0 ± 1.7	20.3 ± 0.4/20.9 ± 0.2	PCR-RFLP	5
Li, 2018 (31)	Case-control study	China	1122 (45.3)	40.9/42.8	55.3 ± 13.3/55.2 ± 10.2	26.1 ± 4.7/25.4 ± 4.6	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry	8
Schou, 2012 (32)	Prospective cohort study	Denmark	10185 (7.8)	45.2/56.6	64 (57-71)/58 (43-69)[2]	28.9 (26.1-32.2)/24.6 (22.3-27.5)[2]	The ABI PRISM 7900HT Sequence Detection System	7
Singh, 2015 (33)	Case-control study	India	590 (47.5)	35.7/35.5	48.5 ± 14.5/49.0 ± 16.2	27.2 ± 3.3 (F),25.1 ± 4.9 (M) /25.9 ± 4.1 (F), 24.8 ± 5.5 (M)	PCR-RFLP	4
Yan 2020 (18)	Case-control study	China	2208(49.2)	51.7/49.1	58.8 ± 9.7/59.2 ± 9.9	26.8 ± 3.5/25.9 ± 3.8	SNaPshot	7

SD, standard deviation; BMI, body mass index; NOS, Newcastle–Ottawa scale; F, female; M, male; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; HRM, high resolution melting.

[1] median (minimum-maximum).

[2] median (interquartile range).

DISCUSSION

The inconsistency in the results regarding the association between the *ABCA1* 69C>T polymorphism and the risk of T2DM underlines the need for a meta-analysis on this topic. Therefore, we performed a meta-analysis including 10 studies and found that *ABCA1* 69TT is associated with a decreased risk of T2DM; this tendency was more pronounced in the Asian population.

ABCA1 is an efficient transporter of cholesterol from the cell to the liver and is highly expressed in β cells (17, 34). The absence of ABCA1 in β cell results in accumulation of cellular cholesterol, reduction in insulin secretion, and progressive impairment in glucose tolerance (17). In this regard, several studies investigating the association between the *ABCA1* polymorphisms and the risk of T2DM have been conducted. A meta-analysis investigating the association between *ABCA1* 219R>K polymorphism and the risk of T2DM revealed that patients with a variant allele had a lower risk of T2DM (35). Another meta-analysis on the association between the *ABCA1* 230R>C and *ABCA1* 69C>T polymorphisms and T2DM showed that these single nucleotide polymorphisms were not associated with increased susceptibility to T2DM (36). However, the aforementioned meta-analysis included data only from three studies; hence, further meta-analyses including recently published studies are warranted.

Cholesterol accumulation in β cells impairs glucose metabolism and reduces insulin secretion (16). Several clinical studies have shown that *ABCA1*C69T is associated with lipoprotein metabolism. Patients with the *ABCA1* 69CC genotype had higher plasma triacylglycerol and very-low-density lipoprotein cholesterol levels than patients with the CT genotype (37). In addition, a study that included 391 Han Chinese adults showed that patients with the *ABCA1* 69CT or TT genotype had 0.68-fold lower risk of non-alcoholic fatty liver disease than those with the CC genotype (38). In line with these studies, our results indicate that the *ABCA1* 69T allele is associated with decreased risk of T2DM.

Because eight of the 10 included studies were conducted in Asian populations, we performed a subgroup analysis for the Asian population. Similar to the overall result, the *ABCA1* 69CC or CT genotype was significantly associated with higher risk of T2DM than the TT genotype in the Asian population; the association size (OR value) was greater than that for the entire study.

Our study has some limitations. First, it was not possible to perform the subgroup analysis for the non-Asian population because only two studies were available. Second, T2DM is a complex and multifactorial disease; therefore, potential gene-gene and gene-environment interactions should be considered. However, insufficient information, including nutrition, lifestyle, and demographic details, precluded further adjustments in the analysis. Third, our meta-analysis had substantial heterogeneity, possibly because of the small number of studies included.

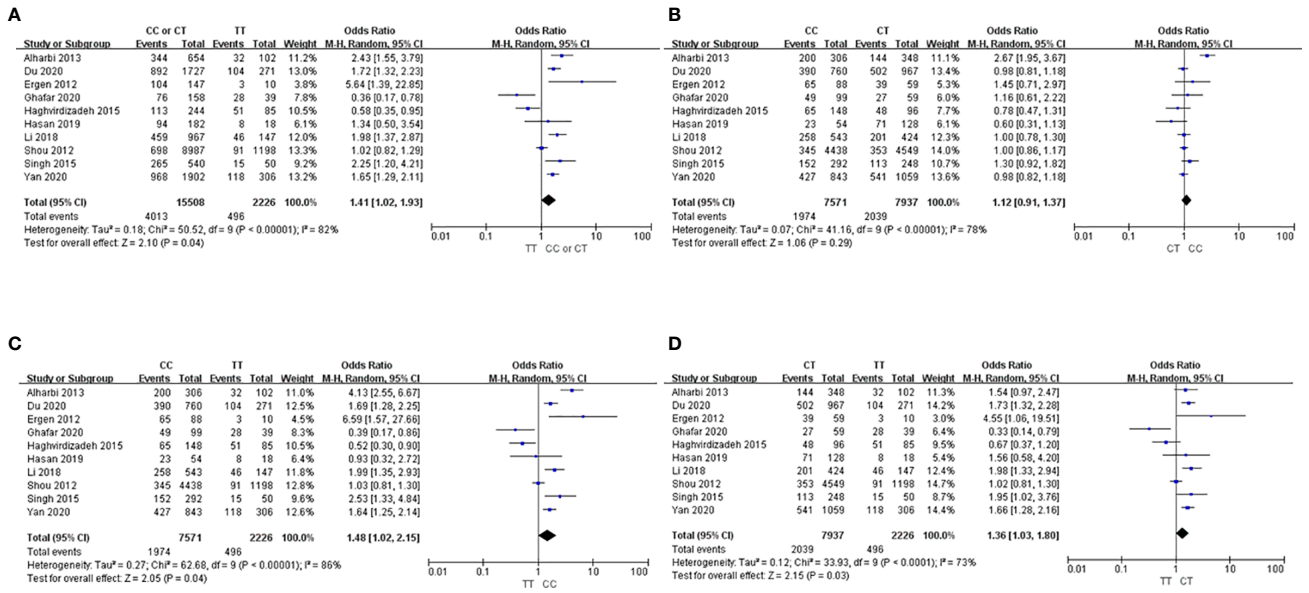

FIGURE 2 | Forest plots showing the association between the *ABCA1* 69C>T genotype and T2DM risk. **(A)** CC or CT *vs.* TT, **(B)** CC *vs.* CT, **(C)** CC *vs.* TT, **(D)** CT *vs.* TT.

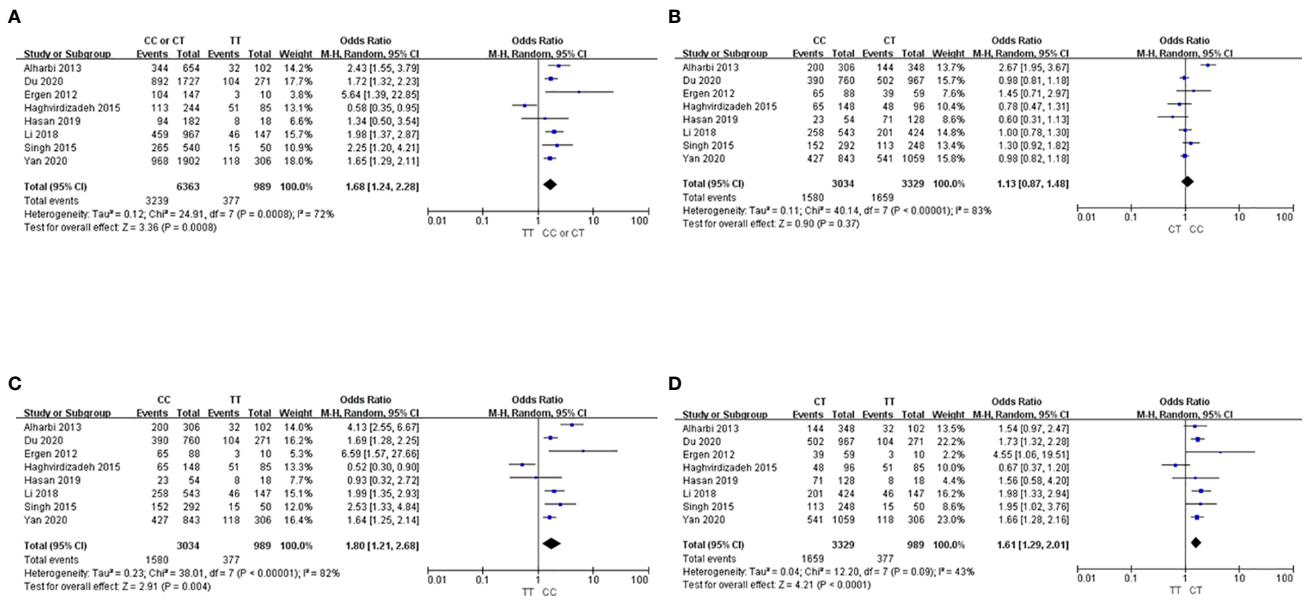

FIGURE 3 | Forest plots showing the association between the *ABCA1* 69C>T genotype and T2DM risk in Asians. **(A)** CC or CT *vs.* TT, **(B)** CC *vs.* CT, **(C)** CC *vs.* TT, **(D)** CT *vs.* TT.

In conclusion, our findings indicate a significant association between the *ABCA1* 69C>T polymorphism and T2DM risk. Large-scale population-based association studies should be conducted to validate the risk indicated by our meta-analysis and investigate potential gene-gene and gene-environment interactions on T2DM risk.

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.

AUTHOR CONTRIBUTIONS

All the authors have made substantial contributions to the conception of the study. HY, ML, and HG contributed to designing the study. HY and ML contributed to acquisition and analysis of data. JY, GS, and HG contributed to interpretation of data. HY and ML contributed to drafting of the manuscript. HG contributed to critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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When Leptin Is Not There: A Review of What Nonsyndromic Monogenic Obesity Cases Tell Us and the Benefits of Exogenous Leptin

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Obesity is a pandemic condition of complex etiology, resulting from the increasing exposition to obesogenic environmental factors combined with genetic susceptibility. In the past two decades, advances in genetic research identified variants of the leptin-melanocortin pathway coding for genes, which are related to the potentiation of satiety and hunger, immune system, and fertility. Here, we review cases of congenital leptin deficiency and the possible beneficial effects of leptin replacement therapy. In summary, the cases presented here show clinical phenotypes of disrupted bodily energy homeostasis, biochemical and hormonal disorders, and abnormal immune response. Some phenotypes can be partially reversed by exogenous administration of leptin. With this review, we aim to contribute to the understanding of leptin gene mutations as targets for obesity diagnostics and treatment strategies.

Keywords: *LEP*, leptin, congenital leptin deficiency, non-syndromic monogenic obesity, metreleptin

INTRODUCTION

Obesity

The equilibrium between the amount of energy intake and expenditure is the key to fat tissue homeostasis. Such an equation is not that simple as it seems. Obesity is a complex disease defined as abnormal or excessive adiposity that may cause different levels of health impairment. In the last two decades, obesity has shown an expressive growth rate, assuming global epidemic proportions (1). To date, the global obesity prevalence is greater than the underweight prevalence (2). Approximately 107.7 million children and 603.7 million adults presented with obesity in 2015. In this same year, high body mass index (BMI) was associated with 4 million deaths (3). Moreover, it was estimated that obesity and overweight will affect 57.8% of the global adult population in 2030 (4).

The increase in the global burden of obesity has been attributed to the interplay between distinct factors, such as environmental factors, e.g., urban progress that generated daily facilitating

mechanisms, more passive forms of entertainment, and reduced physical activity, leading to a spread of sedentary lifestyle (5). Concomitantly, there is an increasing consumption of hypercaloric sweeteners, and fat-rich foods, and an expanding offer of quickly prepared and cheap ultraprocessed foods, differentially affecting persons with respect to their socioeconomic status (6, 7).

Both maternal and paternal BMI are correlated with offspring BMI, supporting family history of overweight/obesity as a risk factor for childhood overweight/obesity (8). Modern obesogenic environments are clearly contributing to the increase of overweight and obesity burden; however, the genetic heritability is a key factor in adiposity (9–12). Common forms of obesity are caused by a combination of environmental factors with many genetic variants. However, monogenic forms were identified in humans, caused by variations in a single gene that can be transmitted autosomically or X-linked (13).

Over the last 20 years, a large number of different rare variants were described in genes encoding proteins within the leptin-melanocortin pathway, involved in hypothalamus cell differentiation, appetite modulation, and energetic metabolism regulation. These may cause early-onset obesity, hyperphagia, and endocrine abnormalities. Mendelian nonsyndromic obesity, alias monogenic nonsyndromic obesity is associated with only 5% of obesity cases in the population. Though rare, it is the most severe form of the disease (6, 14).

LEP was the first gene associated with nonsyndromic monogenic obesity in 1997 (15); since then, with ever refining biomolecular technologies, further rare gene variants have been described (14). *LEP* variants cause the rarest recessive inherited form of the disease, affecting one case in 4.4 million (16), in which congenital leptin deficiency (OMIM#614962) has mostly been reported in consanguineous families (15, 17–24). This specific group of patients has successfully been treated with metreleptin, resulting in a reduction of food intake, body fat mass, and metabolic and endocrine abnormalities (25–27).

In this review, we have mapped the identified patients with *LEP* pathogenic variants the related phenotypes and the benefits of the metreleptin treatment, to broaden our understanding of leptin congenital deficiency and current therapeutic approaches.

THE HYPOTHALAMIC REGION AND LEPTIN SIGNALING PATHWAY

The definition of which brain area is responsible for the control of the energy homeostasis of the body was debated for years until a study by Hetherington and Ranson appeared in 1940 (28). In this work, the researchers introduced lesions in the hypothalamus of rats, which significantly enhanced adiposity. Furthermore, it was observed that these animals showed alterations in the reproductive tracts as well as decreased body growth. These experiments defined the hypothalamus as an area of central importance for energy homeostasis.

The hypothalamus is a small area of the brain located underneath the thalamus and is composed of grey matter which is formed by a conglomeration of neurons organized in nuclear bodies, and white matter, consisting of myelinated neurons. Among other functions, the hypothalamus works as a neuroendocrine circuit that modulates body temperature, electrolyte balance, fertility, sleep-wake cycle, circadian rhythms, thirst, hunger, and energy expenditure (29, 30). The eating behavior and energy expenditure pathways are controlled by an even more complex circuit, including the hypothalamic neuron network and signals from peripheric tissues.

In the decades after the discovery of hypothalamus function, some mutations of rodents were found to cause obesity with comorbidities. The first description by Ingals et al. from the Jackson Laboratories, described a mutation named *ob* (31). In 1965, the mutation *db* was described by another group from the same laboratory (32). In the 1970s, Coleman made the most important discoveries when using parabiosis experiments with both *ob*/*ob* and *db*/*db* mice. He described a soluble factor, produced by the adipose tissue, that would act on the hypothalamus to inhibit food intake and enhance energy expenditure (33). Many years later, the *ob* gene was cloned and the factor was named leptin (34). Leptin was the first of many adipokines to be described and is released by mature white adipocytes. It contributes to the control of the nutritional status of the body (30, 35).

Leptin is mainly secreted by the adipose tissue, crosses the blood-brain barrier, and binds to the leptin receptor b isoform (LRb). LRb is the longest form (1162Aa) from a family of six LR isoforms (LRa-LRf), differing only in the intracellular tail length (36). LRb is mainly expressed in the arcuate nucleus and binding to leptin initiates intracellular auto-phosphorylation of Janus kinase 2 (JAK2), an enzyme that promotes activation of signal transducers and activators of transcription (STATs) (37). In turn, LRb is phosphorylated in three tyrosine residues (Tyr985, Tyr1077, and Tyr1138) (38).

Tyr985 poses a binding site to the src-homology 2 domain protein (SHP2) that modulates extracellular signal-regulated kinase (ERK) 1/2 pathways. JAK2-ERK 1/2 activation was shown to diminish appetite, decrease body weight, and modulate the thermogenic sympathetic outflow (39). Tyr1077 phosphorylates the transcription factor signal transducer-activator of transcription 5 (STAT5) and gives it a role in energy homeostasis; however, it also is an important mediator of leptin action on the reproductive axis (40). Tyr1138 residue coupled to the SH2 domain mediates the activation of the transcription factor STAT3 and its subsequent translocation to the nucleus, upregulating suppressor of cytokine signaling 3 (SOCS3) gene expression, which encodes a leptin signaling inhibitor, that acts by binding to tyrosine residue 985 and JAK2 (38, 41).

The STAT3 transcription factor also inhibits AgRP and NPY gene expression and upregulates POMC (42–44). Furthermore, the LepRb-JAK2-STAT3 signaling pathway was shown to enhance CART expression in the arcuate nucleus, which acts as an anorexigenic appetite controlling neuropeptide, and plays a crucial role in adaptive thermogenesis in response to changes in ambient temperature (45, 46). Concomitantly, JAK2

phosphorylates insulin receptor substrates 1 and 2 (IRS1 and 2), causing the downstream activation of forkhead box protein O1 (FOXO1) and mammalian target of rapamycin (mTOR). The activation of the mTOR pathway by leptin is responsible for the activation of POMC neurons and for the peripheric effects of leptin in leukocytes (47–49). FOXO1 also promotes POMC transcription and AgRP/NPY inhibition (38, 50). In response to leptin, these pathways initiate anorexigenic signaling that leads to NPY and AgRP neuron suppression and release of POMC and CART (**Figure 1**). POMC is a pleiotropic precursor protein that is cleaved in a tissue-specific manner, into peptides and hormones that serve a variety of biological functions, such as pigmentation, adrenocortical function, energy homeostasis, sexual behavior, reward system, and immunity (51). In the arcuate nucleus, POMC is cleaved by prohormone convertase 1/3 (PC1/3) into α-melanocyte-stimulating hormone (α-MSH) (52). The α-MSH produced by POMC and CART neurons activates the melanocortin-4 receptor (MC4R) in the paraventricular nucleus, initiating a satiety signal through the upregulation of brain-derived neurotrophic factor (BDNF). BDNF is a neurotrophin involved in neuronal development, differentiation, and survival (35, 53, 54). Knowledge about the physiological function of CART peptides is limited, due to the lack of characterization of its specific receptor; however, the role of CART peptides has been associated with the regulation of food intake and body weight (55). Additionally, leptin modulates the energy expenditure by stimulating the thermogenesis of the brown adipose tissue and the browning of white adipose tissue (38).

During fasting and decreased energy availability, serum leptin levels are diminished, and the ghrelin hormone is secreted by the stomach. It binds to its receptor at orexigenic neurons of the arcuate nucleus, upregulating NPY and AgRP expression. These neuropeptides act as melanocortin receptor antagonists and promote increased food intake and decreased energy expenditure (35, 56).

CONGENITAL LEPTIN DEFICIENCY

Previously named obese (*ob*) gene, *Lep* and its human homolog gene (*LEP*) was first identified and mapped to human chromosome 7q32.11994 (57). Leptin is an adipocyte-derived hormone that regulates body energy homeostasis by its action in the hypothalamus. The effect of leptin deficiency was first observed in the severely obese (*ob/ob*) mice, which have a leptin mutation, triggering many comorbidities such as obesity, hyperinsulinemia, corticosterone excess, and infertility (57). High levels of corticosterone suppress growth hormone secretion, responsible for disturbing linear growth and severe insulin resistance (58). In addition, these mice develop hypogonadotropic hypogonadism, which leads to their infertility (59).

Different types of *LEP* mutations causing congenital leptin deficiency have been described in different ethnicities (38, 60).

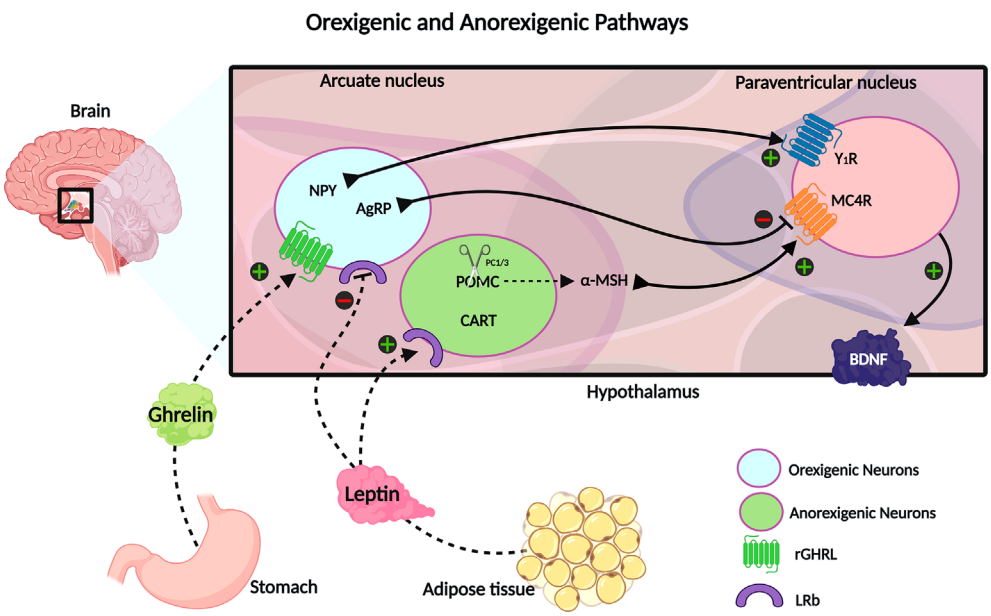

FIGURE 1 | The neuroendocrine circuit regulated by leptin and ghrelin hormones. Ghrelin is secreted by the stomach in response to a decrease of energy stock and upregulates the orexigenic neurons, stimulating the NPY and AgRP signaling, which promotes the increase of energy intake and decreases the energy expenditure. On the opposite, there is the leptin hormone, mainly secreted by fat tissue, and acts in inhibiting orexigenic neurons and upregulating anorexigenic neurons to express CART and POMC; the last one is cleaved into α-MSH. CART and α-MSH potentialize the satiety signal and increase the metabolic rate. Created with Biorender.com.

Individuals carrying only one functional copy of *LEP* exhibit diminished/undetectable serum leptin levels and show normal birthweight followed by rapid weight gain, hyperphagia, hyperinsulinemia, development of type 2 diabetes mellitus, sympathetic system dysfunction, and hypothalamic pituitary gonadal axis dysfunction (23, 61–63). Leptin is also known to be important for the modulation of inflammatory responses (49). Leptin deficiency is related to reduced serine protease inhibitor α1-antitrypsin (A1AT) expression, a neutrophil elastase (NE) inhibitor that protects tissue from inflammatory damage. NE activity is elevated in leptin-deficient subjects. This A1AT/NE imbalance ratio is proposed to affect energy expenditure and promote insulin resistance, obese-related inflammation, and liver steatosis (64). NE overactivity leads to lung tissue impairment and degradation of pulmonary proteins, which may cause chronic obstructive pulmonary disorder and asthma in obese subjects (**Figure 2**) (65). In this review, we mapped all cases of congenital leptin deficiency, describing their clinical phenotypes (**Table 1**) and the results of leptin replacement therapy when available.

LEP Pathogenic Mutations

A total of 67 leptin-deficient cases (52% female) were revised (**Table 2**), including 39 cases of frameshift mutations, four probands with deletion mutations, 20 missense mutation carriers, and three individuals identified with nonsense mutations (**Figures 3** and **4**). The majority of the cases are of Pakistani origin (67%), followed by Turkish origin (9%), Egyptian origin (11% each), Indian, German and Colombian origin (3% each), and of Chinese and Austrian origin (2%). The improvement of clinical parameters of 12 cases was reported after replacement therapy with recombinant methionyl human leptin (r-metHu-Leptin). The probands benefited from amelioration of hyperphagia followed by weight loss, normalization of biochemical parameters, immunophenotype and pubertal development, and cognitive/neuropsychological improvement (18, 19, 27, 66–71).

Individuals With *LEP* Frameshift Mutations

ΔG133 (p.g133_VfsX14)

A frameshift mutation is caused by an insertion or deletion of one or several nucleotides in a DNA coding sequence, causing disrupted codon sequence reading that leads to abnormal protein products. The first variant associated with monogenic nonsyndromic obesity in humans was a frameshift mutation on *LEP* described in 1997 by Montague et al. (1997) (15). Two cousins (Ob1 and Ob2) from a highly consanguineous family of Pakistani descent were homozygous for a deletion of a single guanine nucleotide in codon 133 (ΔG133), leading to the introduction of 14 aberrant amino acids after codon 132 and a premature stop codon. Both individuals had normal birthweight (Ob1: 3.46 kg; Ob2: 3.53 kg) but suffered from increased weight gain and severe obesity from an early age. At the time of the study, Ob1 was an 8-year-old female, weighing 86 kg (>99.6th centile), with a height of 137 cm (75th centile), and percentage body fat of 57%. She suffered from hyperphagia, leg bone

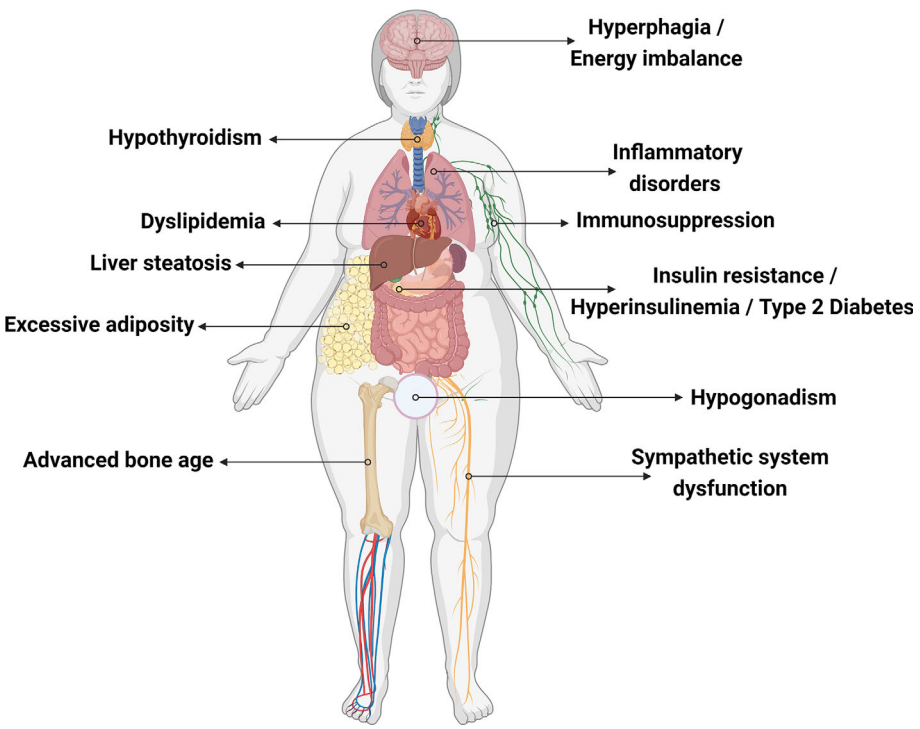

FIGURE 2 | Effects of leptin deficiency on human body. Created with Biorender.com.

TABLE 1 | Prevalence of the clinical phenotypes of the cases.

Clinical phenotype	*N* (%)	Number of cases per type of mutation			
		Frameshift	Deletion	Missense	Nonsense
Hyperphagia	66 (100%)	39	4	20	3
Hyperinsulinemia	25 (38%)	12	1	9	3
Hypothyroidism	6 (9%)	3	2	1	0
Serum lipid disorders	9 (14%)	4	1	4	0
History of inflammatory disorders	6 (9%)	4	0	1	1
$CD4^+$ lymphocytopenia	2 (3%)	2	0	0	0
Advanced bone age	2 (3%)	2	0	0	0
Elevated cortisol	18 (27%)	12	2	4	0
Hypertension	4 (6%)	1	0	3	0
Fatty liver	3 (5%)	0	1	2	0
Elevated liver enzymes	2 (3%)	0	1	1	0
Hyperglycaemic	1 (2%)	0	0	1	0
Amenorrhoeic	4 (6%)	0	0	4	0
Hypogonadotropic hypogonadism	5 (8%)	0	0	4	1
Sympathetic system dysfunction	4 (6%)	0	0	4	0
Acanthosis nigricans	3 (5%)	0	0	3	0

TABLE 2 | Monogenic variants identified in leptin gene.

Type/pathogenic mutations	Exon	*N*	Origin	Consanguinity	Gender	References
Frameshift						
ΔG133 (p.g133_VfsX14)	3	2	Pakistani	Yes	Female	(1)
		1	Pakistani	Yes	Male	(2)
		1	Pakistani	Yes	Female	(3)
		7	Pakistani	Yes	Male	(4)
				No	Male	
				Yes	Female	
				Yes	Female	
				Yes	Female	
				Yes	Male	
				Yes	Male	
		9	Pakistani	Yes	M: 5; F: 4	(5)
		5	Pakistani	Yes	M:1; F: 4	(6)
		12	Pakistani	Yes	M: 4; F: 8	(7)
p.Leu161fsX170	3	1	Pakistani	Yes	Male	(4)
p.Leu12 fs	2	1	Egyptian	N.A.	Female	(8)
Deletion						
p.35delIle	2	1	Pakistani	Yes	Female	(4, 5)
		1	Pakistani	Yes	Female	
c.1-44del42	Intron 1	1	Pakistani	Yes	Male	(7)
Gross deletion		1	N.A.	Yes	Male	(9)
Missense						
p.Arg105Trp	3	3	Turkish	Yes	M: 1; F: 2	(10)
		1	Turkish	Yes	Female	(11)
		1	Turkish	Yes	Male	(12)
		2	Pakistani	Yes	Male	(7)
		1	Egyptian	N.A.	Female	(8)
P.Ile35Ser	2	1	Egyptian	N.A.	Male	(8)
p.Asn103Lys	3	2	Egyptian	Yes	M:1; F: 1	(13)
		2	German	No	M:1; F: 1	(14)
		1	Pakistani	Yes	Male	(15)
p.Leu72Ser	3	1	Austrian	No	Female	(16)
p.His118Leu	3	1	Chinese	N.A.	Male	(17)
p.Asp100Tyr	3	1	Turkish	Yes	Male	(18)
p.Cys117Tyr	3	1	Pakistani	Yes	Male	(7)
p.Asp100Asn	3	1	Indian	Yes	Female	(19)
p.Cys117Phe	3	2	Colombian	Yes	Female	(20)
Nonsense						
p.Q55X	3	1	Indian	Yes	Female	(21)
p.Trp121X	3	2	Egyptian	Yes	M: 1; F:1	(22)

N.A., not available; M, male; F, female.

FIGURE 3 | Localization of the variants on the leptin amino-acid sequence. Created with Biorender.com.

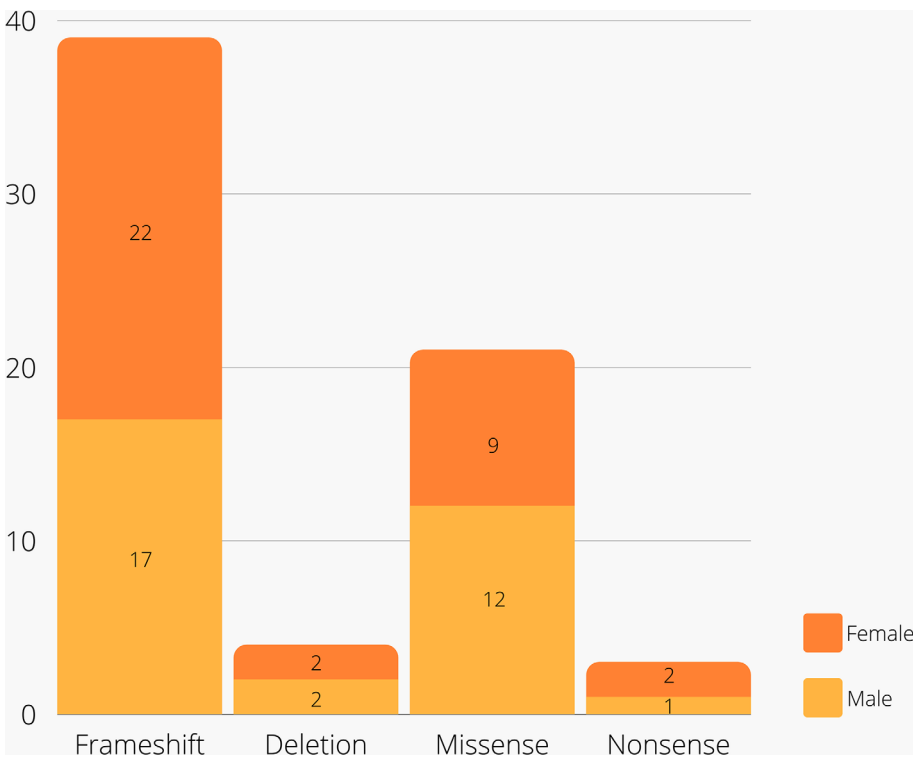

FIGURE 4 | Distribution of types of mutations and gender among cases.

abnormalities, and elevated insulin levels. Ob2 was a 2-year-old male, weighing 29 kg (>99.6th centile), with a height of 89 cm (75th centile), and body fat of 54%. He suffered from hyperphagia and walking difficulties. The probands serum leptin levels were very low, near to the detection limit of the assays.

In 2002, a third leptin-deficient child (Ob3) from a consanguineous family of Pakistani ancestry was identified homozygous to ΔG133 by the same group (18). However, the two families were not related. The third proband was an approximately 3.5-year-old male, weighing 38.8 kg (>98th centile), with a 100-cm height. He had a fat mass of 21.9 kg, body fat of 55.4%, high insulin blood level, and his energy intake was approximately 250 kJ/kg of lean body mass.

Two years later, a fourth pediatric case (Ob4) homozygous for ΔG133 was reported (19). Like that observed in the previous cases, the actual female case was also from a consanguineous Pakistan origin family, not related to the previous families. She had a normal birthweight (3.2 kg), however, due to hyperphagia and rapid weight gain, at the age of 5, her proband weight was 64.4 kg and height was 121.8 cm, reaching a BMI of 43.4 kg/m^2 and a BMI SD score of 7.0. The patient developed asthma at 2 years old. Like that reported in the former cases, plasma leptin levels of the Ob4 case were undetectable. Her total fat mass was 34.3 kg, body fat was 53.4%, and as expected, hyperinsulinemia and high plasma triglycerides were remarkable. The patient also suffered from perineal dermatitis and recurrent hospitalization to treat asthma crisis.

A study conducted by Fatima et al. (2011) (20) included 25 unrelated obese children from Pakistan, in which 18 (76%) were from a consanguineous family. From the total sample, nine children had low or undetectable serum leptin and from these, eight were the result of consanguineous marriages. All cases had hyperphagia, and almost all cases had normal birthweight and started to gain weight before 12 months of life, except one that was overweight at birth. The *LEP* sequence of each case was determined, and seven were identified homozygous for the ΔG133 frameshift mutation, indicating this variant as a founder mutation in Central Punjab. One case was homozygous for a novel frameshift mutation in the exon 3 (p.Leu161fsX170), as a result of CT deletion in codon 161, shifting the stop codon position and disrupting leptin folding sites.

A larger study included a cohort of 62 randomly chosen probands with early onset of severe obesity, also from Central Punjab, Pakistan (72). Homozygous *LEP* variants were identified in 10 (16.1%) unrelated probands from consanguineous families, of these, nine (14.5%) were homozygous carriers for the previously mentioned frameshift mutation (ΔG133). All nine carriers were leptin deficient, and three subjects exhibited hyperinsulinemia. Interestingly, eight out of nine families with ΔG133 frameshift mutation belonged to the Arain community, which has a tradition in consanguineous marriages and is part 1 of the larger subethnic populations of Punjab. Like in the earlier studies, it was concluded that ΔG133 may be a founder mutation. In another study by Saeed et al. (2014) (73), two unrelated families from Pakistan were enrolled, accounting for five ΔG133 homozygous and eight ΔG133 heterozygous probands. Ten volunteers without LEP mutations were used as control group. As expected, the homozygous probands had undetectable plasma leptin levels, and the mean fasting and postprandial insulin levels were higher than in the heterozygous or control groups. It was further shown that leptin-deficient probands had a sustained relatively low ghrelin levels, without postprandial reduction. This work also demonstrated that heterozygosity for the *LEP* mutation did not produce partial leptin deficiency.

Another work, also performed by Saeed et al. (2015) (21), extended the evaluation to a cohort of 76 severely obese unrelated consanguineous probands from Pakistan. From these, 12 probands (21%) were ΔG133 carriers, four male and eight female probands, with ages of >1–13 years (median: 1.2 years) old. As in the previous study by Saeed et al. (2014) (73), the frameshift variant carriers also belong to the Arain community, reinforcing the hypothesis that ΔG133 is a founder mutation.

p.Leu12fs

The most recent cross-sectional study conducted by ElSaeed et al. (2020) (22) revealed a new frameshift mutation p.Leu12fs, caused by a single cytosine nucleotide deletion at position 34 in exon 2. This new variant was identified in a 10-year-old girl, with normal birthweight. The patient showed rapid weight gain and became obese at the age of 8 months. Her weight was 80 kg, height was 128.2 cm, and her BMI was 48.7 kg/m^2. Low leptin level reached 0.9 ng/dl.

Individuals Homozygous for *LEP* Deletion Mutations

p.35delIle

Deletion mutations can affect a single nucleotide, large sequences, or whole chromosomes. The aforementioned study carried out with Pakistan infants by Fatima et al. (2011) (20), also described a deletion of mutation in one proband. They observed deletions of thymine and cytosine from codon 35 and adenine from codon 36. As a result, one codon was deleted from exon 2 and an isoleucine amino acid was removed from the leptin protein N-terminus. The patient was a 7-month-old female, weighing 14.8 kg (BMI >95th percentile), with moderate leptin deficiency of 3.6 ng/ml, lower than normal levels. This deletion was also identified in another infant patient from Pakistan (72): a 1.5-year-old girl with a BMI of 27 kg/m^2, hyperphagia, and undetectable leptin, unlike the first case with the same deletion and slightly decreased leptin levels.

c.1-44del42

A novel mutation caused by a 42-pb deletion in intron 1 was identified by Saeed et al. (2015) (21) in a male proband 1.5 years old from a Pakistani origin. This deletion results in an abnormal splicing and disrupts the expression of exon 2. The serum leptin was not detectable, and cortisol level was increased.

Complete Deletion of *LEP* Exons 2 and 3

The only case of large deletion in the *LEP* human gene was identified in a 6-month-old boy, with consanguineous parents.

The patient was severely obese with remarkable hyperphagia and low leptin serum level. Additionally, the patient presented with central hypothyroidism, elevated liver enzymes, dyslipidemia, and grade 2 hepatic steatosis. The electrophoresis revealed the lack of a fragment of 2,862 bp in the PCR product of the patient. This missing fragment corresponds to exons 2 and 3 of *LEP*. These findings indicate a homozygous deletion of the leptin coding sequences in an infant proband (74).

Individuals Homozygous to *LEP* Missense Mutations

p.Arg105Trp

The first missense mutation associated with high BMI and low leptin serum level was a cytosine to thymine substitution at codon 105, exon 3, promoting the arginine to tryptophan amino acid change in the protein (24). This homozygotic mutation (p.Arg105Trp) was observed in three obese Turkish patients (referred as 14, 24, and 31) with marked hyperphagia and elevated insulin plasma levels. This was the first description in adult probands, which allowed the observation of the effects of leptin deficiency on reproductive function. Patient 14, a 34-year-old female, had primary amenorrhea. Patient 24, a 22-year-old male with normal karyotype, showed clinical features of hypogonadotropic hypogonadism and did not enter puberty. These observations are in line with the *ob/ob* mouse phenotype. Using transfected cells with the mutant *LEP* cDNA, it was observed that the mutant protein is synthesized, however, not secreted.

From the same family, another female was reported. She had severe obesity and amenorrhea and was homozygous to p.Arg105Trp (patient 40). Novel information about patients 14, 24, and 31 were also later reported (23). Patient 14 that had amenorrhea until March 1998, entered puberty, but her mammary gland consisted mainly of adipose tissues. Patient 40 was 30 years old and presented with abnormal menstrual function, with a menstrual period of about 8 months, since she was 29 years old. Patient 31, a homozygous 7-year-old female, showed subclinical hypothyroidism and low total T-cell counts. Sympathetic system dysfunction was observed in all patients. Of note, 11 individuals of this family were described with obese phenotype; however, only the four probands cited above were alive. The other seven probands died during childhood due to infections. Statistical analysis revealed an increased mortality during childhood for this type of mutation (23).

Another case reported from that family revealed a homozygous mutation of the *LEP* gene (p.Arg105Trp) (71). The proband was a 7-year-old boy that started to gain excessive weight at 3 months of age. At 5 years old, he was introduced to leptin replacement therapy (leptin replacement therapies and their benefits will be discussed in the sections below). Before the therapy and despite obesity, the patient did not exhibit other comorbidities than hyperinsulinemia and hyperphagia. His BMI was 39.6 kg/m^2. The patient's general cognitive ability and neuropsychological function were analyzed using DAS and NEPSY scores, respectively, and were lower than age-matched controls (71).

The genetic screening of 73 children with early-onset obesity and hyperphagia from Pakistani consanguineous families identified 14 subjects carrying *LEP* variants, including two siblings homozygous to p.Arg105Trp. A third affected sibling died at the age of 3. The two alive probands were 1.5- and 10-year-old males and were the first Pakistani identified with this leptin variant (21). This pathogenic variant was also identified in a 3-year-old Egyptian female, who presented with the classical features of leptin deficiency, with a normal birthweight of 3 kg, but expressive weight gain leading to obesity after 5 months. Her BMI was 29.4 kg/m^2, and she exhibited high blood pressure, hyperphagia, aggressive behavior when demanding food, and very low leptin level (0.1 ng/dl) (22).

p.Ile35Ser

The study performed by ElSaeed et al. (2020) (22) also described a missense mutation in exon 2. The amino acid change isoleucine to serine at codon 35 was identified in a 7-month-old boy, with a BMI of 28.8 kg/m^2, high blood pressure, and hyperphagia. Analysis revealed that this variant was likely to be disease causing.

p.Asn103Lys

A novel leptin missense in homozygosigotic mutation caused by a C-to-A substitution in the third base of codon 103 (p.Asn103Lys), located in the protein N-terminus region was described in two Egyptian siblings, a 3-year-old boy and a 7-year-old girl. Both had early-onset severe obesity, remarkable hyperphagia, hyperinsulinism, and low leptin serum levels. The probands were the result of consanguineous marriage, in which the parents were heterozygous variant carriers. The probands had a history of delayed development, but no other clinical features to suggest syndromic obesity (75). This variant was also observed in two German siblings, however, in this case, from parents without known consanguinity (27). As observed in previous leptin-deficiency cases, both had normal birthweight, presented with hyperphagia, and with rapidly increased weight. Interestingly, the German siblings exhibited high serum leptin levels (>50 ng/ml), differing from the aforementioned Egyptian siblings carrying this same variant. The authors investigated HEK293 cells transfected with *LEP* p.Asn103Lys and wild type and concluded that the leptin mutant is secreted, however, not functional (27).

The presence of p.Asn103Lys leptin mutation was also investigated in the Pakistani population since Pakistan is the 9th country among 188 countries in obesity ranking. A case-control observational study enrolled 475 unrelated subjects, of which 250 were obese. The homozygous mutation was identified in a 10-year-old boy from a highly consanguineous family with several cases of obesity. The proband had the classical features observed in obesity caused by congenital leptin deficiency, with leptin level close to limit detection. The authors then hypothesized that this mutation, besides processing inactive leptin, also may be associated with low leptin serum levels (76).

p.Leu72Ser

A 14-year-old girl from Austrian origin, with mild obesity, was identified with a homozygous transition in exon 3 (thymine to cytosine), resulting in leucine to serine exchange in codon 72 of leptin protein. She presented with undetectable leptin, and remarkable clinical history of rapid weight gain leading to obesity after normal birthweight, even after restricted caloric

diet. She was the offspring of healthy and nonconsanguineous parents heterozygous to this mutation. The proband entered puberty but exhibited features of hypogonadotropic hypogonadism. She had hyperinsulinemia, increased transaminases, and dyslipidemia in addition to sympathetic system dysfunction observed under cold pressor test. No immune abnormalities were observed, with normal T-cell counts and activity. Function assays proved that Leu72Ser leptin variant is expressed but not secreted; however, in contrast to other cases with congenital leptin deficiency, this patient only showed mild obesity, probably due to a residual leptin activity or due to the patient's daily environment, favorable to controlling energy intake since infancy (77).

p.His118Leu

The first study to identify a leptin mutation in obese patients from the Han Chinese population screened the *LEP* coding region of 35 obese cases with BMI ≥32 kg/m² and controls with BMI <25 kg/m². The His118Leu mutation in exon 3 of *LEP* was described for the first time in one obese patient with a BMI of 46.0 kg/m². In addition to obesity, he presented with hypertension, metabolic syndrome, fatty liver, sleep apnea, gastric ulcer, and chronic superficial gastritis. The current mutation was accessed using the predicting bioinformatic tools PolyPhen2 and SIFT, which classified the mutation as "damaging." Pedigrees and anthropometric and biochemical data regarding the patient were not available, and functional analyzes of this novel variant were not performed. Further studies to address these limitations are required (78).

p.Asp100Tyr

Another missense *LEP* mutation was identified in a Turkish boy, whose parents were healthy first-degree cousins. The child was born with normal weight but had rapid weight gain in the postnatal period, reaching a BMI of 38.6 kg/m² at 2.5 years of age. Despite a history of recurrent ear and pulmonary infections, the patients' T-cell counts and function were normal. His leptin serum level was high, suggesting the presence of a variant disturbing leptin activity. Leptin receptor mutations were ruled out by sequencing, and an Asp to Tyr exchange at codon 100 of *LEP* was identified. Functional analysis was conducted using HEK23 cells expressing mutant and wild-type leptin. The results indicated that the novel leptin variant is expressed and secreted but fail to induce Stat3 phosphorylation in its receptor, so, the p.Asp100Tyr change results in a nonfunctional hormone (79).

p.Cys117Tyr

The study by Saeed et al. (2015) (21) also identified a substitution of guanine by adenine at position 350 of the *LEP* coding sequence, resulting in p.Cys117Tyr change in the leptin protein. The homozygous carrier was a 1.5-year-old male, obese, and with undetectable leptin. Using SIFT and PolyPhen software, the novel variant was predicted to impair protein function.

p.Asp100Asn

A case report of an Indian infant at 10 months of age described classical features of congenital leptin deficiency, including low serum leptin concentrations. She was a child of healthy, nonobese, consanguineous parents. Genetic analysis identified an amino acid change of asparagine to aspartic acid in position 100 (p.Asp100Asn) of the protein. Prediction effect analysis classified this novel variant as "probably pathogenic" and "pathogenic" by PolyPhen-2 and by SIFT, Log ratio test, and MutationTaster, respectively (80).

p.Cys117Phe

Two extremely obese Colombian sisters with consanguineous parents were identified with early-onset obesity, pointed toward obesity with a genetic cause. Genomic DNA sequencing of *LEP* identified both sisters homozygous for a novel missense mutation in codon 117, a cysteine to phenylalanine substitution. This was the first leptin variant reported on the American Continent. The older sister was 24 years old and had primary amenorrhea. At 16 years old, her BMI reached 53 kg/m², and bariatric surgery was performed, resulting in a weight loss of 20 kg, which was not sustained. She presented with increased weight gain in the following 5 years, even under restricted sugar and fat diet and 60 min of walk daily. The younger sister was 21 years old, with primary amenorrhea in addition to hypertriglyceridemia, insulin resistance, and *acanthosis nigricans*. Both patients exhibited breasts and genitals at Tanner Stage V, and undetectable leptin serum levels (81).

Individuals Homozygous for *LEP* Nonsense Mutations

p.Q55X

One variant of nonsense mutation was identified in an Indian girl, daughter of consanguineous parents. She was 8 years old, had a BMI of 52.9 kg/m², and presented with hyperinsulinemia. After syndromic obesity was discarded by lacking dysmorphic features, leptin serum levels were measured and were very low. Her *LEP* gene was screened and a novel nonsense mutation was identified in codon 55 of exon 3, resulting from a C>T substitution at position 163 of the coding sequence (p.Q55X). Her parents were heterozygous for this mutation (82).

p.Trp121X

Another nonsense mutation, also in exon 3, was present in two Egyptian siblings with obesity, from consanguineous parents. Both children showed undetectable leptin plasma levels. The eldest child was a 13.5-year-old boy with a BMI of 49.7 kg/m², and the younger was a 2-year-old girl with a BMI of 42.5 kg/m². Molecular analysis identified both homozygous for the nonsense mutation p.Trp121x (c.223G>A). History of respiratory tract infection was reported to the male proband. It was also reported that two younger siblings, also with severe obesity and with recurrent respiratory infection, had died. The parents were heterozygous carriers (83).

The Emergence of Recombinant Methionyl Human Leptin (r-metHu-Leptin or Metreleptin) Replacement Therapies and Their Benefits

Studies with administration of recombinant leptin in mice began in 1995 and showed that its use ameliorated their obesity through reduction of food intake and higher energy expenditure (84–86).

The administration of leptin also reverted the hyperinsulinemia, corticosterone levels, and infertility problems related to the mutated mice (87). Leptin-deficient mice have growth problems and severe insulin resistance, while humans with leptin deficiency do not have growth restriction and moderate insulin resistance (**Table 3**) (66, 88). These studies were the basis for the development of human treatment with recombinant leptin.

The replacement therapy with metreleptin in the Ob1 patient, one of the first individuals identified homozygous to ΔG133, was applied (66). The synthetic leptin dose was calculated based on age, gender, and body composition. It was administrated 0.028 mg/kg of lean mass of Ob1 daily at 8 a.m. per 12 months. The dose was equivalent to 10% of the predicted Ob1 normal leptin concentration. Weight loss was noted within 2 weeks of treatment and was sustained during all periods of treatment, resulting in a decrease of weight by 16.4 kg. Leptin replacement also resulted in reduced food consumption, which was taken as the main cause of weight loss. There was increased in physical activity as a result of the improvement of mobility. The patient that was prepubertal before therapy had a gradual increase in her basal and stimulated serum follicle-stimulating hormone and luteinizing hormone concentrations during the treatment. In the 12th month, the patient showed features of early puberty, with a pulsatile nocturnal pattern of gonadotropin secretion. After 2 years of treatment, she was 11 years old and showed pubertal development, with the growth of uterus and ovaries, visible follicles on ultrasound, and regular menstrual cycles. Tanner III stage was reached when she was 13.6 years old (18).

Later in 2002, a longer study of leptin replacement therapy, ranging from 10 to 50 months, included the Ob1, Ob2, and Ob3 patients (18). As observed in the first Ob1 report, daily subcutaneous injection of r-metHu-Leptin induced weight loss in all patients after 2 weeks of treatment. It was accompanied by reduction of plasma insulin and a reduction in total serum cholesterol and increase of serum HDL cholesterol. Marked reduction in energy intake was observed after 2 months of treatment, confirmed by parental reports and an *ad libitum* test meal. Before treatment, Ob2 and Ob3 patients exhibited CD4+ lymphopenia and deficient lymphocyte activity; however, the therapy with the synthetic leptin addressed these issues, and both patients showed normal immunophenotype.

The fourth case carrying the ΔG133 variant (Ob4) also was benefited from leptin replacement therapy. The patient showed some episodes of weight gain, which was contoured with leptin dose adequacy. Ob4 had a decrease of total fat mass of 15.9 kg with 48 months of therapy, and BMI lowered to 24.2 kg/m^2. Biochemical parameters and immune function were improved, with attention to the amelioration of perineal dermatitis, asthma symptoms, and fewer occurrences of urinary tract infections. These results are in line with the observations from all previous cases homozygous for ΔG133 after r-metHu-Leptin therapy. The authors concluded that the abnormal thyroid function of Ob4 was completely normalized after leptin replacement and T4 therapy withdrawal (19).

Three adult probands from the Turkish family identified with the p.Arg105Trp mutation (23, 24) went through r-metHu-Leptin therapy. They presented a strong effect on fat mass, hyperphagia, hypothalamic-pituitary-gonadal axis, cholesterol levels (67), and improved neuroplasticity (68). After 18 months, the patients lost 60.0, 76.2, and 47.5 kg, respectively. The hypogonadism reported in the male patient was reversed, noted by the appearance of puberty features, as acne; facial, axillary, and pubic hair; development of sexual organs; and ejaculation. The same was observed for the female patients who had regular menstrual periods and ovulation after therapy. The eldest female had type 2 diabetes mellitus, which was controlled during the course of leptin replacement. The brains of the three patients were analyzed using structural magnetic resonance images, in the period prior to the therapy and after the exogenous leptin administration. An increase in the concentration of the gray matter tissue was detected in the regions of the anterior cingulate gyrus, inferior parietal lobule, and cerebellum, which are associated with the regulation of hunger and satiation. The effect of stimulus with food images in the patient's brains was also evaluated using functional magnetic resonance imaging (69). Leptin replacement diminished the rate of self-reports of hunger after they were exposed to food images.

The effects of leptin replacement on eating behavior were accessed by Farooqi et al. (2007) (70) in two leptin-deficient patients, a male aged 14 years and a 19-year old female. Both were treated with r-metHu-Leptin for 7 days, resulting in a decrease of energy intake of 88 kJ/kg for the male and 71 kJ/kg of for the female, during an *ad libitum* test meal. To examine the brain activity of the probands before and after therapy, functional magnetic resonance imaging was used. The patients were shown images of food and nonfood during fasting and after feeding and were asked to pick the images they liked. Leptin replacement was associated with a decreased "liking" rate from patients who were shown images of food. Before the leptin treatment, the accumbens-caudate was activated either during the starving state and feeding state. After the therapy, accumbens-caudate activation was found to be correlated only with the starving state, suggesting a role for leptin in food reward perception and satiety signaling.

In light of the findings above, Paz-Filho et al. (2008) (71) aimed to elucidate whether or not a therapy with synthetic leptin plays a role in cognitive development. The study was performed with a 7-year-old boy with congenital leptin deficiency. The neurocognitive evaluation started before the therapy, when the boy was 5 years old, and then followed up after the beginning of leptin replacement. The Differential Ability Scales (DAS) and subtests from the NEPSY test were used in this assessment. The patient's general cognitive ability increased concomitantly to the therapy, as well as his neuropsychological functions. The patient's parents reported emotional problems and behavioral regulation that was within the normal limits observed in age-matched controls. Additionally, hyperinsulinemia was reversed, accompanied by a lower calorie intake and weight decrease and cholesterol amelioration.

Finally, Wabitsch et al. (2015b) (27) reported that two p.Asn103Lys carriers exhibited significant amelioration of

TABLE 3 | Benefits of the metreleptin therapy.

Mutation—case	Initial metreleptin dose	Adjustments/reason?	Duration	Outcomes	Ref.
ΔG133—Ob1	0.028 mg/kg LBW	Yes. Changes in the body weight	12 months	**(1)** Loss of 16.4 kg of fat mass; **(2)** sustained reduction of energy consumption; **(3)** increase of physical activity; and **(4)** increase of serum gonadotropin.	(23)
ΔG133—Ob2	0.017 mg/kg LBW	Yes. Changes in the body weight/ metreleptin neutralization by antibodies	36 months	**(1)** Loss of 10.7 kg of fat mass; **(2)** reduction of energy consumption; **(3)** decrease of plasma insulin level, serum cholesterol, triglycerides, and LDL and increase of serum HDL cholesterol; and **(4)** normalization of the immunophenotype	(2)
ΔG133—Ob3	0.014 mg/kg LBW	Yes. Changes in the body weight/ metreleptin neutralization by antibodies	6 months	**(1)** Loss of 2.2 kg of fat mass; **(2)** Reduction of energy consumption; **(3)** decrease of plasma insulin level, serum cholesterol, triglycerides, and LDL and increase of serum HDL cholesterol; and **(4)** normalization of the immunophenotype.	(2)
ΔG133—Ob4	0.019 mg/kg LBW	Yes. Changes in the body weight	48 months	**(1)** Loss of 15.9 kg of fat mass; **(2)** normalization of plasma triglycerides, insulin, and TSH and increase of HDL cholesterol; **(3)** increase of white blood cells count, with amelioration of perineal dermatitis and asthma.	(3)
p.Arg105Trp—male	0.01–0.04 mg/kg	Yes. Changes in the body weight	18 months	**(1)** Loss of 52.1 kg of fat mass; **(2)** resolution of the hypogonadism; and **(3)** neuroplasticity.	(24–26)
p.Arg105Trp—female	0.01–0.04 mg/kg	Yes. Changes in the body weight	18 months	**(1)** Loss of 37.6 kg of fat mass; **(2)** resolution amenorrhea; and **(3)** neuroplasticity.	(24–26)
p.Arg105Trp—eldest female	0.01–0.04 mg/kg	Yes. Changes in the body weight	18 months	**(1)** Loss of 39.1 kg of fat mass. **(2)** resolution of the type 2 diabetes mellitus and amenorrhea; and **(3)** neuroplasticity.	(24–26)
Leptin-deficient—male	N.A.	N.A.	7 days	**(1)** Decrease of hyperphagia and **(2)** increase of satiety.	(27)
Leptin-deficient—female	N.A.	N.A.	7 days	**(1)** Decrease of hyperphagia and **(2)** increase of satiety.	(27)
p.Arg105Trp—7-year-old boy	1.36 mg/day	Yes. Changes in the body weight	28 months	**(1)** Weight loss; **(2)** amelioration of hypertension, dyslipidemia and hyperinsulinemia; and **(3)** increase of patient's general cognitive ability and neuropsychological function.	(12)
p.Asn103Lys	0.03 mg/kg LBW	No	2 months	**(1)** Amelioration of hyperphagia and satiety and **(2)** weight loss of 6.2 kg.	(14)
p.Asn103Lys	0.03 mg/kg LBW	No	2 months	**(1)** Amelioration of hyperphagia and satiety and **(2)** weight loss of 3.5 kg.	(14)

LWB, lean body mass.

hyperphagia and satiety, leading to weight loss when treated daily with leptin (0.03 mg/kg of lean mass).

Taken together, these studies showed that the use of recombinant leptin improves the clinical spectrum of leptin-deficient patients. The use of the exogenous leptin results in quality-of-life improvements.

CONCLUSION

From the *LEP* gene discovery in 1994 to the application of exogenous leptin to overcome congenital leptin deficiency, much progress has obviously been made. This adipokine has a unique neuroendocrine role regulating energy expenditure, food consumption, and the hypothalamic-pituitary-gonadal axis. Homozygous *LEP* autosomal recessive mutations are correlated to the expression of disease phenotypes including hyperphagia, hyperinsulinemia, immune system dysfunction, and infertility. Since the description of the first monogenic variant, a total of 17 mutations in 67 cases were reported in the literature, with the majority reported from highly consanguineous families (20, 27, 77). After the expression of recombinant leptin in 1995 and description of the human mutations, leptin replacement was established as a new therapy to treat congenital leptin deficiency. This therapeutic approach was capable of diminishing hyperphagia, resulting in weight loss; normalization of insulin plasma concentrations, cholesterol, thyroid function, and pubertal development; and improvement of immune system, reducing the occurrence of infections (18, 19, 23, 24, 27, 66–68, 70). Additionally, exogenous leptin induced an increase of gray matter tissue, which was associated with reduced energy intake (69). The current therapy may also improve cognitive impairments and neurophysiologic dysfunctions (71). This review highlights the importance of a molecular diagnosis of leptin gene expression in severe obesity. *LEP* mutations are extremely rare; therefore, it is inefficient to include *LEP* in the genetic screening routine. The gene product however is at the core of body energy homeostasis. It is therefore possible that mutations that contribute to mild phenotypes have more severe outcomes depending on genetic or environmental factors. Through observations of the clinical phenotype, bioactive leptin measurement (16), and genetic counseling, the patient could benefit from the most appropriate clinical management and treatment, improving his health and overall quality of life.

AUTHOR CONTRIBUTIONS

KS, AF, PB, and FK: conception and design of the study and drafting the article. KS and JM: review of the literature. KS: wrote the manuscript. VZ, FK, CM, PB, JC, CM-M, and AF: revised the manuscript critically for important intellectual content. All authors read and approved the final version.

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Molecular Characterization of an Aquaporin–2 Mutation Causing Nephrogenic Diabetes Insipidus

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The aquaporin 2 (AQP2) plays a critical role in water reabsorption to maintain water homeostasis. AQP2 mutation leads to nephrogenic diabetes insipidus (NDI), characterized by polyuria, polydipsia, and hypernatremia. We previously reported that a novel *AQP2* mutation (G215S) caused NDI in a boy. In this study, we aimed to elucidate the cell biological consequences of this mutation on AQP2 function and clarify the molecular pathogenic mechanism for NDI in this patient. First, we analyzed AQP2 expression in Madin-Darby canine kidney (MDCK) cells by AQP2-G215S or AQP2-WT plasmid transfection and found significantly decreased AQP2-G215S expression in cytoplasmic membrane compared with AQP2-WT, independent of forskolin treatment. Further, we found co-localization of endoplasmic reticulum (ER) marker (Calnexin) with AQP2-G215S rather than AQP2-WT in MDCK cells by immunocytochemistry. The functional analysis showed that MDCK cells transfected with AQP2-G215S displayed reduced water permeability compared with AQP2-WT. Visualization of AQP2 structure implied that AQP2-G215S mutation might interrupt the folding of the sixth transmembrane α-helix and/or the packing of α-helices, resulting in the misfolding of monomer and further impaired formation of tetramer. Taken together, these findings suggested that AQP2-G215S was misfolded and retained in the ER and could not be translocated to the apical membrane to function as a water channel, which revealed the molecular pathogenic mechanism of AQP2-G215S mutation and explained for the phenotype of NDI in this patient.

Keywords: aquaporin 2, nephrogenic diabetes insipidus, water reabsorption, polydipsia, hypernatremia

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INTRODUCTION

Nephrogenic diabetes insipidus (NDI) is characterized by impaired arginine vasopressin (AVP)-induced water reabsorption in the kidney, leading to polyuria, polydipsia, and hypernatremia. The most severe outcomes include impaired mental development, dilation of the urinary tract, and death (1–3). NDI can be secondary to other clinical conditions, such as drugs (e.g., lithium and cisplatin

therapy) and electrolyte abnormalities, or caused by mutations in the vasopressin V2 receptor (*AVPR2*, OMIM#304800) or *AQP2* (OMIM#125800, 107777) (4–7). AVPR2 accounts for X-linked cases of NDI. Current therapeutic options for congenital NDI focus on ameliorating symptoms rather than curing, which are limited and only partially effective (3).

AQP2 is a key factor for maintaining normal body water homeostasis. When the plasma osmolality increases, antidiuretic hormone (AVP) is released from the pituitary gland and binds to AVPR2 in principal cells of the kidney collecting duct, resulting in the accumulation of AQP2 in the apical plasma membrane, which is responsible for water reabsorption (8, 9). AQP2 forms a homotetramer in the plasma membrane, and each monomer is composed of 271 amino acids, containing six transmembrane spanning regions with the intracellular COOH terminus, which is essential for correct routing of AQP2. AVP increases phosphorylation of AQP2 at ser256 and ser269, which is important for the accumulation of AQP2 (10–12). K63-linked ubiquitylation of lys270 is critical for the internalization and degradation of AQP2 from the plasma membrane (13). Up to now, there were 65 mutations of *AQP2* reported to cause NDI, and missense/nonsense mutation is the most common mutation type (14). More than 90% of mutations are inherited in autosome recessive mode, which can be categorized into three types, depending on the structural analysis: (i) the pore features (e.g. A70D), (ii) the tetramer assembly (e.g. T126M), and (iii) the monomer folding (e.g. A47V). The remaining 10% of mutations are inherited in a dominant trait, involving the C-terminal tail for AQP2 routing (e.g. R254L) (15–19). Most autosomal recessive cases had severe phenotypes in contrast to autosomal dominant NDI (20).

We previously reported a homozygous missense mutation AQP2-G215S (substitution of Gly215 with Serine) caused NDI in a boy for the first time (21). The initial symptom occurred at 4 months, and the male patient had a total urine volume greater than 4 L in 24 h when diagnosed. Sequence alignment of AQP2 proteins indicated Gly215 showed a 100% conservation among six different species. In the study, we aimed to elucidate the cell biological consequences of this mutation on AQP2 function and clarify the molecular pathogenic mechanism for NDI in this patient.

MATERIALS AND METHODS

Cell Culture

MDCK cell line was obtained from Cell Resource Center, Basic Medicine Institute, Chinese Academy of Medical Sciences. MDCK cells were cultured in Eagle's Minimum Essential Medium with 10% fetal bovine serum. All experiments were performed with the approval of the institutional review board and ethics committee of PUMCH, and written informed consents were obtained from the patients.

Plasmid Construction

Coding sequence of human AQP2 was cloned into a vector containing the pCMV6 promoter. Mutant AQP2-G215S plasmid was constructed by site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kits, Agilent) and confirmed by sequence analysis.

Electroporation

Cells were resuspended in 100 µl buffer, and 2 µg plasmid was added. Cells were transferred to a sterile 0.2-cm cuvette (Cell Line Nucleofector™ Kit L, Lonza) and electroporated using Lonza® Nucleofector® II electroporation system according to the manual and protocol. After transfection, cells were gently resuspended in pre-warmed medium. Indomethacin (5×10^{-5} M, Selleck) was added to culture medium 24 h after electroporation and incubated overnight. The medium was replaced with fresh medium containing indomethacin (5×10^{-5} M, Selleck) plus forskolin (5×10^{-5} M, Selleck) or not and incubated for 2 h. Then, the cells were harvested for protein extraction or fixed for immunocytochemistry.

Western Blot

Total cellular proteins were extracted using RIPA buffer containing protease and phosphatase inhibitors. Total membrane and cytoplasmic membrane protein were extracted by a kit (Minute™ Plasma Membrane Protein Isolation and Cell Fractionation Kit, invent BIOTECHNOLOGIES) according to the protocol. All procedures were performed on ice, and protease and phosphatase inhibitors were added into buffer A before use. 20 to 50×10^6 cells were prepared for plasma membrane protein isolation. Equal amounts of protein were loaded on a 12% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dry milk for 2 h, followed by incubation with primary antibodies (AQP2 polyclonal antibody, 1:1000, cell signaling technology, #3487; Cadherin antibody, 1:1000, Cell Signaling Technology, ab16505; Calnexin, 1:1000, Thermo Fisher Scientific, MA3-027) over night. Membranes were washed and incubated with HRP-conjugated goat anti-rabbit IgG. Bands were visualized by enhanced chemiluminescence (Pierce™ ECL Western Blotting Substrate kit, Thermo Fisher Scientific).

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde in PBS (pH 7.4) and blocked with 5% normal goat serum and 0.2% Triton X-100. Primary antibodies, including Calnexin (Thermo Fisher Scientific), were diluted in 5% normal goat serum and incubated overnight. Alexa Fluor 594- and Alexa Fluor 488-labeled secondary antibodies were used. Samples were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted on glass slides using the ProLong antifade kit (Thermo Fisher Scientific).

Transcellular Osmotic Water Permeability Measurements

Cells derived from 0.33 cm^2 of confluent monolayers were seeded onto 0.33-cm^2 polycarbonate filters (Costar, Cambridge, U.S.A.). On the second day after seeding, the medium was aspirated and replaced by fresh medium in the presence of indomethacin (5×10^{-5} M, Selleck) to reduce basal intracellular cyclic adenosine monophosphate (cAMP) levels. Three days after seeding, osmotic water transport was assayed in the presence of indomethacin with or without adding forskolin (5×10^{-5} M, Selleck), by incubation

of the apical compartment with 150 μl of 0.5× KHB (1× KHB contains 1.2 mM $MgSO_4$, 128 mM NaCl, 5 mM KCl, 2 mM NaHPO4, 10 mM NaAc, 20 mM HEPES, 1 mM $CaCl_2$, 1 mM L-alanine, 4 mM L-Lactate; pH=7.4), containing 30 mg/L phenol-red and the addition of 800 μl KHB to the basal compartment. After incubation for 2 h at 37°C, the content of the apical compartment was mixed with a pipette, and two aliquots of 50 μl per insert were put into Eppendorf tubes and diluted to 600 μl with Tris-buffered saline (TBS: 20 mM Tris, 73 mM NaCl; pH=7.6) containing 1% (w/v) extrane (Merck, Darmstadt, Germany). After mixing and centrifugation, absorbance at 479 nm was measured. The osmotic water transport (Pf) was calculated from the acquired absorbances as described (22, 23).

Visualization of AQP2 Structure

To visualize the potential impact of the G215S mutation, we built the crystal structure of G215S tetramer using homology modelling with Prime (24). The template structure was human Aquaporin 2 (PDB ID: 4NEF) with 99% sequence identity (17). The snapshots were prepared using VMD 1.9.3 (25).

Data Analysis and Statistics

All results represented at least three independent replications. All data were represented as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (San Diego, California USA, www.graphpad.com). Differences between groups were analyzed as appropriate using *t* test or one-way ANOVA and post-hoc Tukey's multiple comparison tests. $P < 0.05$ (two tailed) was considered to be statistically significant.

RESULTS

Expression of AQP2-G215S Was Decreased in the Cell Membrane Compared With AQP2-WT

We expressed AQP2-WT and AQP2-G215S in MDCK cells by electroporation with plasmid, a cell line demonstrated to be a typical model for AQP2 function (26). Since AQP2 is a membrane protein and functions as a water channel in the apical membrane of principle cell, we further isolated the cytoplasmic membrane and total membrane and detect AQP2 expression by WB (as shown in **Figure 1**). The results showed that the expression of AQP2 was similar in the total membrane of AQP2-WT-transfected cells and AQP2-G215S-transfected cells (p=0.44). However, AQP2 was decreased in the cell membrane of AQP2-G215S–transfected cells compared with AQP2-WT-transfected cells (p<0.01), even after treated with forskolin (p<0.001), suggesting that mutant AQP2-G215S may not be transported to the cell membrane.

AQP2-G215S Is Retained in Endoplasmic Reticulum

We further analyzed the subcellular localization of AQP2-G215S in MDCK cells by immunocytochemistry. MDCK cells were electroporated with AQP2-WT or AQP2-G215S plasmid, then fixed and stained to detect the expression of AQP2 and ER marker (Calnexin). Our results showed co-localization of AQP2 with Calnexin in AQP2-G215S-transfected cells compared with AQP2-WT-transfected cells with or without the stimulation of Forskolin (**Figure 2**), which suggested that AQP2-G215S was retained in endoplasmic reticulum, in contrast to AQP2-WT.

AQP2-G215S Displays Impaired Transcellular Osmotic Water Permeability

We analyzed the transcellular osmotic water permeability of AQP2-G215S- and AQP2-WT-transfected MDCK cells. The osmotic water transport (Pf) of AQP2-WT-transfected cells was higher than AQP2-WT–transfected cells (24 ± 2.2 *vs* 5.5 ± 1.0 μm/s, $p<0.01$) (**Figure 3**). With the stimulation of forskolin, the Pf of AQP2-WT-transfected cells was increased (57.8 ± 5.1 μm/s, $p<0.001$), whereas the Pf of AQP2-G215S did not change apparently and remained a much lower level than AQP2-WT ($p<0.001$). These results indicated that the permeability of MDCK cells transfected with AQP2-G215S was impaired, which further supported retention of AQP2-G215S mutant in endoplasmic reticulum.

Structural Visualization of AQP2–G215S

Gly215 was located at alpha-helix in the sixth transmembrane spanning of AQP2 monomer as shown in **Figures 4A, B**. Amino

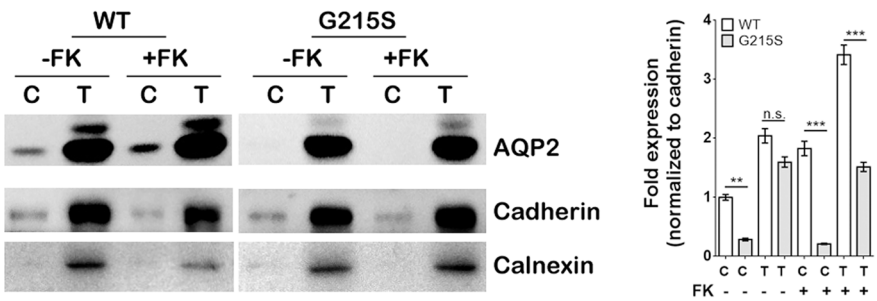

FIGURE 1 | Expression of AQP2-G215S was decreased in the cell membrane compared with AQP2-WT. MDCK cells were transfected with AQP2-WT and AQP2-G215S plasmids. Total membrane proteins (labeled as T) and cytoplasmic membrane proteins (labeled as C) were harvested for AQP2, Pan-cadherin, and calnexin immunoblotting. Pan-Cadherin is the membrane marker, and calnexin is the endoplasmic marker. Protein fold expression normalized to cadherin is shown. WT, wild type; FK, forskolin. Data were shown as mean ± SEM. n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s.=no statistically significant difference.

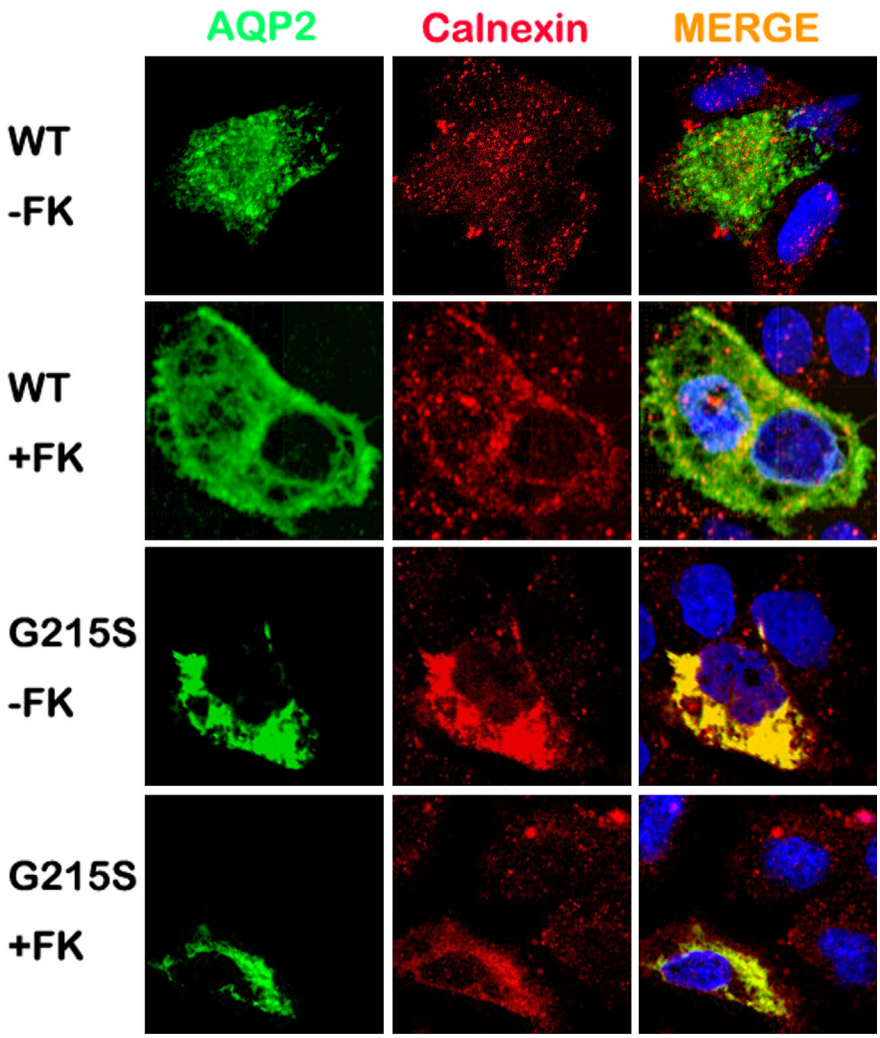

FIGURE 2 | AQP2-G215S was retained in endoplasmic reticulum. MDCK cells were electroporated with AQP2-WT and AQP2-G215S plasmids and stained for AQP2 and endoplasmic reticulum marker (Calnexin). WT, wild type; FK, forskolin.

acids surrounding S215 were on the same monomer, including V142, L143, and L139, suggesting that G215S mutation mostly destabilized AQP2 monomer by changing interactions within intra-monomer helices (**Figure 4C**). Further, G215S, introducing a polar side chain of S215 into a hydrophobic environment, was thermodynamically unfavorable (**Figure 4D**). Based on these information, the serine substitution at Gly215 probably interrupted folding of the sixth transmembrane α-helix and/or the packing of α-helices, resulting in the misfolding of monomer, which may further influence the formation of functional AQP2 tetramer.

DISCUSSION

In this study, we aimed to elucidate cell biological consequences of a G215S mutation in AQP2 that was discovered in a boy with severe NDI (21). The AQP2 mutation was inherited from his parents, who had a heterozygous genotype and normal phenotypes, which supported an autosomal recessive inheritance model. There was a variety of cell lines and animal models applied to clarify molecular action of AQP2 and the pathogenic mechanism of NDI. MDCK, a kidney epithelia cell line, has been widely used as a model to study NDI, because AQP2 transfection reconstituted vasopressin-regulated transcellular osmotic water transport in principle cells of human renal collect duct (26–28). Here, we first constructed AQP2-G215S and AQP2-WT plasmids and analyzed the expression of AQP2-G215S and AQP2-WT by Western blot. Results showed that there was no difference of AQP2 in total membrane. AQP2 forms a homotetramer in the plasma membrane for water reabsorption, so we further isolated cell membrane of MDCK cells and found there was significantly decreased AQP2 expression in cell membrane of AQP2-G215S-

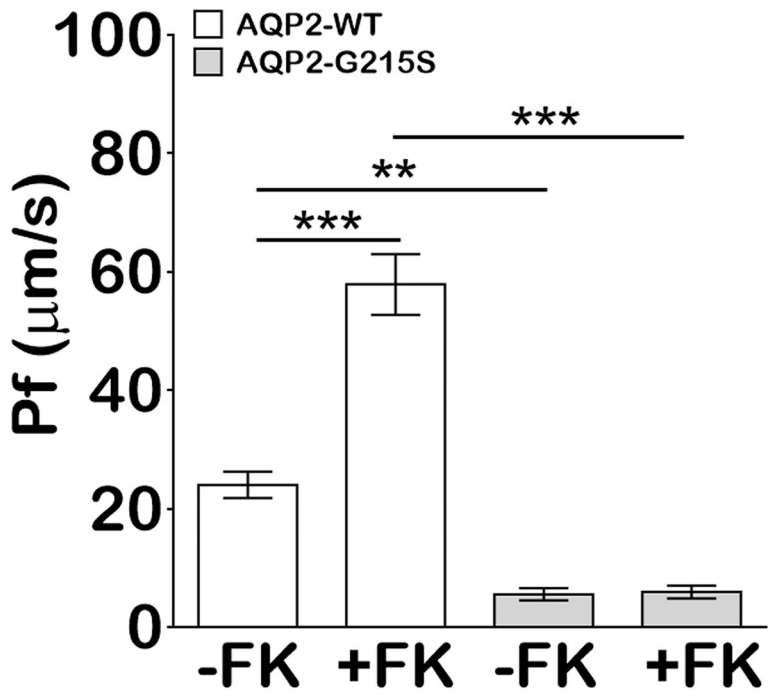

FIGURE 3 | MDCK cells transfected with AQP2-G215S displayed impaired transcellular osmotic water permeability. Cells were seeded onto 0.33 cm^2 polycarbonate filters, and replaced with fresh medium in the presence of 5×10^{-5} M indomethacin. Osmotic water transport was assayed with or without 5×10^{-5} M forskolin. The osmotic water transport (Pf) of AQP2-G215S-transfected and AQP2-WT-transfected cells was calculated as described in Material and Methods. WT, wild type; FK, Froskolin. Data were shown as mean ± SEM. n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$.

transfected cells compared with AQP2-WT-transfected cells, which suggested that AQP2-G215S could not be translocated to cell membrane.

AQP2 monomer is a glycosylated membrane protein with six-pass-transmembrane domain, which is folded and assembled in the ER. During the processing, high-mannose sugar moieties are attached to Asn123 of AQP2, which is part of a canonical N-glycosylation consensus site (N123-X-T125), and the high-mannose sugar groups are removed in the Golgi complex en route to the plasma membrane (29, 30). To investigate the subcellular localization of AQP2-G215S and AQP2-WT in MDCK cells, immunocytochemistry was performed, and results showed co-localization of ER marker (Calnexin) with AQP2-G215S rather than AQP2-WT in MDCK cells with or without the stimulation of forskolin. These results suggested that the AQP2-G215S was retained in the ER, which was also reported in a previous study. For example, David et al. demonstrated that AQP2-F204V mutant was retained in the ER in a renal cell line and *in vivo* (31). Nannette also reported that most AQP2 missense mutants in recessive NDI are retained in the ER (32). There were other studies showing misrouting of AQP2 to Golgi complex (33) or late endosomes or lysosome (34). Here, we demonstrated mutant protein, AQP2-G215S, was retained in the ER for the first time. Functional analysis further demonstrated reduced water permeability in AQP2-G215S-transfected cells compared with AQP2-WT–transfected cells, which further supported that AQP2-G215S mutation was retained in the ER. We further performed structure analysis of AQP2-G215S mutant, and results showed that serine substitution at Gly215 probably interrupted the folding of the sixth transmembrane α-helix and/or the packing of α-helices, resulting in the misfolding of monomer, which may further influence the formation of functional AQP2 tetramer. There were also other studies aiming to elucidate the structural basis for mutations of human aquaporins. For instance, Luisa Calvanese built a 3D molecular model for AQP mutants and explored the effect of mutations on structural feature, which provided a rationale for interpreting mutations. Their results suggested that S216P caused impaired monomer folding, similar to G215S. Taken together, these results showed that AQP2-G215S mutant may be misfolded and retained in the ER and could not be translocated to the apical membrane to function as a water channel. There were limitations in our study, for example, cell biological consequences of this mutation were only investigated in MDCK cell line. Besides, novel therapy is further expected to improve the prognosis of NDI. Up to now, some chemical chaperones were shown to correct the trafficking and folding defects of AQP2 mutants and show efficacy in NDI treatment, and gene editing may correct such mutation and cure diseases in the future (35)

In conclusion, we investigated cell biological consequences of a novel mutation (AQP2-G215S) discovered in a boy with NDI. Results showed that AQP2-G215S mutant may be misfolded and retained in the ER and could not be translocated to the apical membrane to function as water channel. This knowledge elucidated the potential molecular mechanism for NDI in this patient.

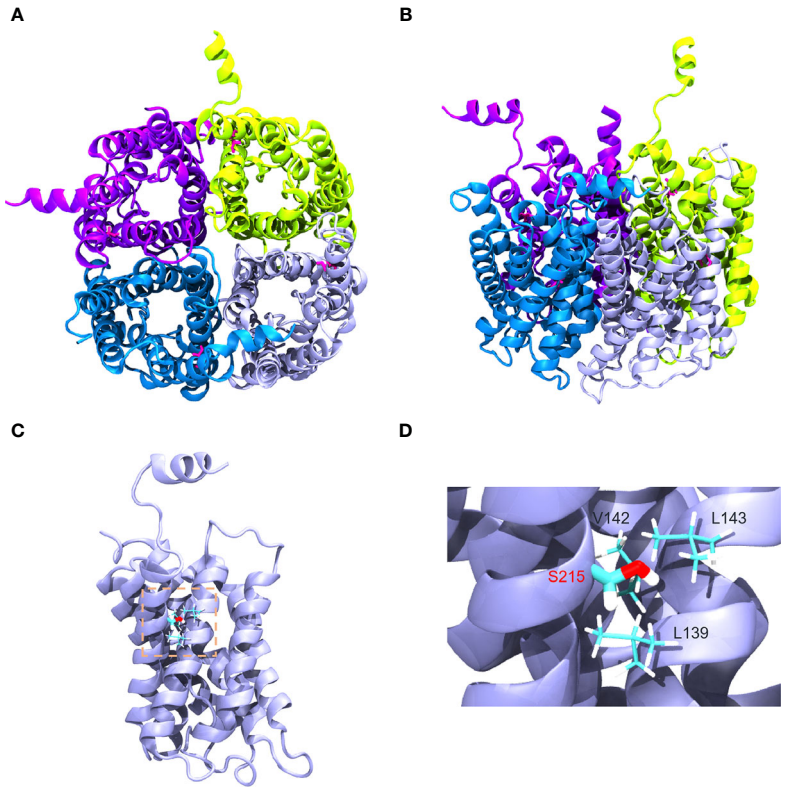

FIGURE 4 | Structural visualization of AQP2–G215S. **(A, B)** The structure of AQP2 (PDB ID is 4NEF) was visualized as a tetramer. **(C, D)** Gly215 was indicated as red in the sixth membrane spanning alpha-helix of the AQP2 monomer as shown.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

Study design: FG, JZ, and WX. Study conduct: QL, BL, JY, and CL. Data collection: QL, BL, JY, and CL. Data analysis: QL, BL, JY, CL, YL, HC, NL, LD, FG, JZ, and WX. Data interpretation: QL, BL, JY, CL, YL, HC, NL, LD, FG, JZ, and WX. Drafting manuscript: QL. Revising manuscript content: QL, BL, JY, CL, HC, NL, LD, FG, JZ, and WX. Approving final version of manuscript: QL, FG, JZ, and WX. All authors contributed to the article and approved the submitted version.

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REVIEW
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Molecular and Cellular Bases of Lipodystrophy Syndromes

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Lipodystrophy syndromes are rare diseases originating from a generalized or partial loss of adipose tissue. Adipose tissue dysfunction results from heterogeneous genetic or acquired causes, but leads to similar metabolic complications with insulin resistance, diabetes, hypertriglyceridemia, nonalcoholic fatty liver disease, dysfunctions of the gonadotropic axis and endocrine defects of adipose tissue with leptin and adiponectin deficiency. Diagnosis, based on clinical and metabolic investigations, and on genetic analyses, is of major importance to adapt medical care and genetic counseling. Molecular and cellular bases of these syndromes involve, among others, altered adipocyte differentiation, structure and/or regulation of the adipocyte lipid droplet, and/or premature cellular senescence. Lipodystrophy syndromes frequently present as systemic diseases with multi-tissue involvement. After an update on the main molecular bases and clinical forms of lipodystrophy, we will focus on topics that have recently emerged in the field. We will discuss the links between lipodystrophy and premature ageing and/or immuno-inflammatory aggressions of adipose tissue, as well as the relationships between lipomatosis and lipodystrophy. Finally, the indications of substitutive therapy with metreleptin, an analog of leptin, which is approved in Europe and USA, will be discussed.

Keywords: lipodystrophy, insulin resistance, diabetes, adipose tissue, genetics, senescence, lipomatosis, immunity

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INTRODUCTION

Lipodystrophy syndromes are rare diseases characterized by generalized or segmental lack of adipose tissue, and by insulin resistance-related metabolic complications such as diabetes, hypertriglyceridemia, hepatic steatosis, and ovarian hyperandrogenism in women. Besides their different clinical presentation with generalized or partial lipoatrophy, accompanied or not by fat overgrowth in other body areas, lipodystrophy syndromes are highly heterogeneous diseases in several other aspects. Specific subtypes of lipodystrophy are associated with additional clinical signs and complications, with, among others, neurological or cardiovascular involvement, showing that

lipodystrophy syndromes are frequently complex multisystem diseases (1–3). The onset of lipodystrophy may be precocious, in early infancy, or delayed in late childhood or adulthood. This review will mainly focus on genetic forms of lipodystrophies. Other forms of lipodystrophies, that will not be covered by this review, result from iatrogenic therapies and/or other environmental factors. This is the case for HIV-related lipodystrophies, due to multifactorial mechanisms resulting, among others, from HIV infection and antiretroviral agents (4). Glucocorticoid therapy leads to body fat redistribution and insulin resistance (5). The identification of causative pathogenic variants in more than 20 genes leading to monogenic forms of lipodystrophies has highlighted several determinants of adipose tissue pathophysiology. This field of research, still highly productive, indicates adipose tissue as a major actor to ensure proper whole-body insulin sensitivity (3, 6).

MAIN MOLECULAR CAUSES OF LIPODYSTROPHY SYNDROMES AND THEIR IMPACT ON ADIPOSE TISSUE FUNCTIONS AND INSULIN RESPONSE

Main Molecular Causes of Lipodystrophy Syndromes

Lipodystrophy syndromes include different congenital to adult-onset diseases, with either generalized or partial lipoatrophy. More than 20 genes are involved in monogenic lipodystrophy syndromes (6–8). Although lipodystrophy syndromes have been considered as ultra-rare diseases, with a prevalence of less than 5 cases per million (9), they are largely underdiagnosed, and systematic genetic screening suggests that 1/7000 individuals could be affected, with a majority of partial forms (10). **Table 1** indicates the main monogenic lipodystrophy syndromes, their specific phenotypic features and the main functions of involved genes. The diversity of molecular causes of lipodystrophy reflects both clinical heterogeneity and close pathophysiological relationships of these diseases. Indeed, beyond the diversity of clinical forms, lipodystrophy syndromes share adipose tissue dysfunction as a key pathophysiological feature, with gene pathogenic variants mostly affecting adipocyte development, differentiation and/or functions (**Figure 1**).

Congenital generalized lipodystrophy syndromes (CGL or Berardinelli-Seip Congenital Lipodystrophy) are autosomal recessive diseases, mainly observed in patients from consanguineous families. They are mainly due to null variants in *AGPAT2* encoding 1-acylglycerol-3-phosphate-O-acyltransferase 2, involved in triglyceride and phospholipid synthesis, or in *BSCL2* encoding seipin, an endoplasmic reticulum membrane protein which contributes to lipid droplet biogenesis (11–16). CGL3 and CGL4 are due to genetic defects in caveolin-1 or cavin-1 respectively, involved in the formation of cell plasma membrane microdomains called caveolae, that initiate several signaling pathways. Caveolin-1 and cavin-1 are also localized at the adipocyte lipid droplet and contribute to intracellular fluxes of lipids (17, 18) (**Figure 2**). Most familial partial lipodystrophies (FPLD) are transmitted as autosomal dominant diseases due to loss-of-function or dominant negative mutations, with initial clinical manifestations occurring from late childhood onwards. Apart FPLD1, which is probably a multigenic form of lipodystrophy syndrome (19), FPLD2 due to *LMNA* pathogenic variants, is the most frequent form of genetically determined partial lipodystrophy (10). Generalized or partial lipoatrophy is a clinical feature of several diseases with accelerated ageing called progeroid syndromes (**Table 1**).

Lipodystrophy and Lipotoxicity

Lipoatrophy is a main contributor of metabolic complications associated with lipodystrophy syndromes. Adipocytes represent 20 to 40% of the cell population of adipose tissue and 90% of adipose tissue volume (20, 21). They arise from mesenchymal stem cells, adipocyte differentiation being carried out under the control of several adipogenic transcription factors. PPARγ is a major adipogenic factor, which regulates the expression of several genes of lipid metabolism and modulates both insulin sensitivity and secretory functions of adipocytes (21, 22). White adipocytes are the most abundant adipocytes in humans. They are characterized by a single voluminous lipid vacuole surrounding a neutral lipid core mainly composed of triglycerides and cholesterol esters. The adipocyte lipid droplet is coated with a monolayer of phospholipids and with proteins belonging to the perilipin family, which play important structural and functional roles. Indeed, white adipocytes have a crucial role in the regulation of energy balance and systemic metabolic homeostasis. In response to nutritional and hormonal signals, the adipocyte lipid droplet is able to store excess energy as triglycerides in the postprandial state, and then to release fatty acids from triglycerides, providing energy substrates for other organs. Adequate storage of energy in adipocytes protects other organs from lipotoxicity due to lipid overload, which leads to oxidative stress, mitochondrial dysfunction, apoptosis and tissue dysfunction (23). In lipodystrophy syndromes, the very limited adipose tissue expandability, due to the major decrease in the capacity of adipose tissue to store lipids, exposes non-adipose organs to lipotoxicity even in well-balanced diet conditions. This results in muscle insulin resistance due to disruption of insulin signaling (24), in non-alcoholic fatty liver disease, as well as in myocardial, endothelial and pancreatic beta-cell dysfunction (25–27). Adipocytes are also important autocrine, paracrine, and endocrine cells that produce numerous adipokines. Among others, leptin regulates satiety by acting on hypothalamic neurons, and modulates carbohydrate and lipid metabolism by acting on muscle, liver adipose tissue and pancreatic beta-cells (28, 29). Integrated effects of leptin ensure an efficient protection of non-adipose tissues from lipid accumulation (30). Adiponectin produced by adipose tissue increases fatty acid oxidation and glucose transport in muscle, and decreases hepatic gluconeogenesis. In lipodystrophy syndromes, the lack of functional subcutaneous fat drives multiple metabolic alterations resulting from altered metabolic and secretory functions of adipocytes. Decreased adipose tissue expandability, and leptin deficiency induce ectopic accumulation of fat upon increased energy intake, even during the

TABLE 1 | Main forms of monogenic lipodystrophy syndromes.

TYPE OF LIPODYSTROPHY	TRANSMISSION	SPECIFIC FEATURES ASSOCIATED WITH LIPODYSTROPHY	GENE INVOLVED, MAIN CELLULAR FUNCTIONS
		Generalized lipodystrophy syndromes	
CGL1	AR	Lytic bone lesions, cardiomyopathy	*AGPAT2*: AGPAT2, synthesis of adipocyte triglycerides and glycerophospholipids
CGL2	AR	Intellectual deficiency, cardiomyopathy, rare neurological signs (encephalopathy, spasticity)	*BSCL2*: seipin, formation of adipocyte lipid droplet
CGL3	AR	Short stature, megaesophagus	*CAV1*: caveolin-1, intracellular transduction pathways, lipid droplet wall
CGL4	AR	Muscular dystrophy, cardiac conduction abnormalities, achalasia	*CAVIN1*: cavin-1, partner of caveolin-1
Autoinflammatory lipodystrophy (JMP, CANDLE)	AR	Fever, muscle atrophy, systemic skin and joint inflammation	*PSMB8*: Immunoproteasome subunit PSMB8, regulation of interferon production, and protein quality control Genes encoding other immuno-proteasome subunits have also been involved
		Partial lipodystrophy syndromes	
FPLD2	AD	Cushingoid facies, possible association with skeletal and cardiac muscular dystrophy	*LMNA:* lamin A/C, structure and functions of nucleus
FPLD3	AD	Severe hypertension	*PPARG:* PPARγ, adipocyte differentiation
FPLD4	AD	Acromegaloid features	*PLIN*: perilipin-1, structure and function of lipid droplet
FPLD5	AR	–	*CIDEC:* CIDEC, structure and function of lipid droplet
AKT2- linked lipodystrophy	AD	Insulin-resistant diabetes with moderate lipodystrophy	*AKT2:* Akt2, insulin signaling intermediate
		Partial lipodystrophy syndromes with pseudo-lipomatosis/Launois-Bensaude multiple lipomatosis	
FPLD6 (*LIPE*-linked lipodystrophy)	AR	Upper-body fat overgrowth (pseudo-lipomatosis), lipoatrophy of limbs, insulin resistance-related traits, muscular atrophy in some cases	*LIPE:* Hormone-sensitive-lipase, release of fatty acids from stored triglycerides in adipocytes and release of cholesterol from cholesterol esters in steroidogenic tissues
***MFN2*-linked lipodystrophy**	AR	Pseudo-lipomatosis, lipoatrophy of non-lipomatous regions, axonal polyneuropathy	*MFN2:* Mitofusin-2, mitochondrial fusion
		Progeroid lipodystrophies	
Hutchinson-Gilford progeria syndrome	*De novo*	Progeria: generalized lipoatrophy, growth retardation, dysmorphic signs, alopecia, bone and skin abnormalities, severe atherosclerosis in childhood	*LMNA:* lamin A/C, structure and functions of nucleus
Progeroid laminopathies	AD or *de novo*	Lipodystrophy with progeroid signs	*LMNA:* lamin A/C, structure and functions of nucleus
Type A mandibuloacral dysplasia	AR	Partial lipodystrophy with progeroid signs and mandibular involvement	*LMNA:* lamin A/C, structure and functions of nucleus
Type B mandibuloacral dysplasia	AR	Generalized lipodystrophy with progeroid signs and mandibular involvement	*ZMPSTE24:* ZMPSTE24/FACE1, post-translational prelamin A maturation
Neonatal progeroid syndrome	AR or *de novo*	Generalized lipoatrophy, progeroid signs, other signs depending on the gene affected	*LMNA, ZMPSTE24:* maturation of prelamin A*CAV1:* caveolin-1, intracellular transduction pathways, structure of lipid droplet
Werner syndrome (adult onset progeria)	AR	Cataracts, trophic skin disorders, cancers, subcutaneous lipoatrophy and increased perivisceral fat	*WRN:* WRN, DNA helicase, DNA repair
MDPL (Mandibular hypoplasia, Deafness and Progeroid features syndrome with Lipodystrophy)	*De novo*	Subcutaneous lipoatrophy and increased perivisceral fat, acro-osteolysis, mandibular and clavicular dysplasia, deafness	*POLD1:* DNA polymerase delta 1, catalytic subunit, DNA replication and repair
***NSMCE2*-linked lipodystrophy**	AR	Short stature, hypogonadism, extreme insulin resistance	*NSMCE2*: E3 SUMO-protein ligase NSE2, genome maintenance, DNA repair
***EPHX1*-linked lipoatrophy**	AR	Generalized lipoatrophy, dysmorphic and progeroid signs, hepatic cytolysis, sensorineural hearing loss	*EPHX1:* Epoxyde hydrolase 1, hydrolysis of epoxides to less-reactive diols

Type 1 Familial Partial Lipodystrophy (FPLD1) is a multigenic form of lipodystrophy syndrome with exacerbated android morphotype and predominant limb lipoatrophy.
AD, autosomal dominant; AGPAT2, 1-Acylglycerol-3-Phosphate-O-Acyltransferase 2; AR, autosomal recessive; CGL, congenital generalized lipodystrophy; JMP, Joint contractures, Muscular atrophy, Microcytic anemia and Panniculitis-induced lipodystrophy syndrome; CANDLE, Chronic atypical neutrophilic dermatosis with lipodystrophy and elevated temperature syndrome; FPLD, familial partial lipodystrophy.

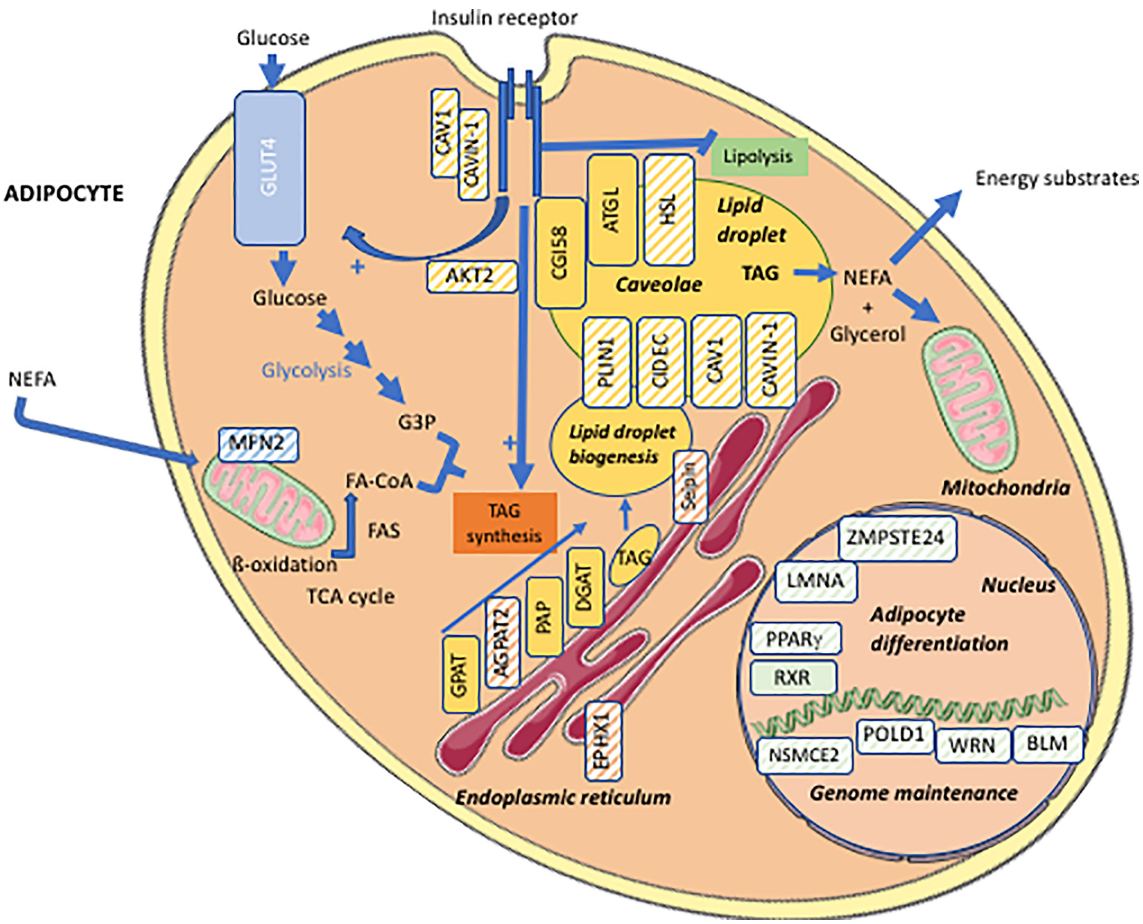

FIGURE 1 | Cellular targets of the main molecular defects responsible for lipodystrophy syndromes. Adipocyte schematic representation with localization of the main proteins involved in the molecular pathophysiology of lipodystrophy syndromes (hatched symbols). AGPAT2, 1-acylglycerol-3-phosphate-O-acyltransferase 2; AKT2, serine/threonine-protein kinase 2; ATGL, adipose triglyceride lipase; BLM, Bloom syndrome protein; CAV1, caveolin-1; CAVIN1, cavin-1; CGI58, comparative gene identification-58, also known as α/β-hydrolase domain-containing 5 (ABHD5); DGAT, diacylglycerol acyltransferase; EPHX1, epoxide hydrolase 1; FA, fatty acid; FA-CoA, fatty acid-coenzyme A; FAS, fatty acid synthase; G3P, glycerol-3-phosphate; GLUT4, glucose transporter 4; GPAT, glycerol-3-phosphate acyltransferase; HSL, hormone-sensitive lipase; LMNA, lamin A/C; MFN2, mitofusin-2; NEFA, non-esterified fatty acids; NSMCE2, E3 SUMO-protein ligase NSE2; PAP, phosphatidic acid phosphatase; PLIN1, perilipin-1; POLD1, DNA polymerase delta 1, catalytic subunit; PPARγ, peroxisome proliferator-activated receptor gamma; RXR, retinoid X receptor; TAG, triacylglycerol; TCA cycle, tricarboxylic acid cycle; WRN, WRN RecQ like helicase.

physiological postprandial state (**Figure 2**). Furthermore, decreased serum levels of adiponectin, although not to the same extent in the different forms of lipodystrophy (31), contribute to insulin resistance and hepatic steatosis associated with lipodystrophy (32). Altered production of other adipokines by lipodystrophic adipose tissue has also been described in several studies, mostly related to HIV-related forms of lipodystrophy (4). It could lead to adipose tissue inflammation and fibrosis, and to a state of systemic low-grade inflammation with insulin resistance (33). In lipodystrophy syndromes, insulin signaling pathways are strongly impacted by mechanisms linked to cellular lipotoxicity and metabolic inflexibility (23, 24). Increased lipid fluxes activate hepatic production of very-low-density lipoproteins, triglycerides and glucose, and impair muscular glucose uptake (6, 34, 35). Leptin deficiency also increases appetite, which worsens metabolic profile. In some forms of partial lipodystrophies, redistribution of body fat, with increased visceral fat and decreased subcutaneous fat, particularly of the lower limbs, also contributes to metabolic dysfunction. Indeed, subcutaneous adipose tissue is physiologically more sensitive to insulin. It has been shown that subcutaneous adipose tissue of the lower part of the body is protective against diabetes and cardiovascular diseases in the general population (36, 37). Conversely, visceral adipose tissue is more sensitive than subcutaneous adipose tissue to adipocyte lipolysis. Furthermore, visceral adipose tissue directly releases fatty acids into the portal vein during lipolysis, which are captured by the liver, leading to an increased risk of liver lipotoxicity, liver steatosis and insulin resistance (**Figure 2**). Visceral fat is more susceptible to chronic inflammation and fibrosis, and produces lower amounts of leptin than subcutaneous tissue (38). Excess visceral, but not subcutaneous

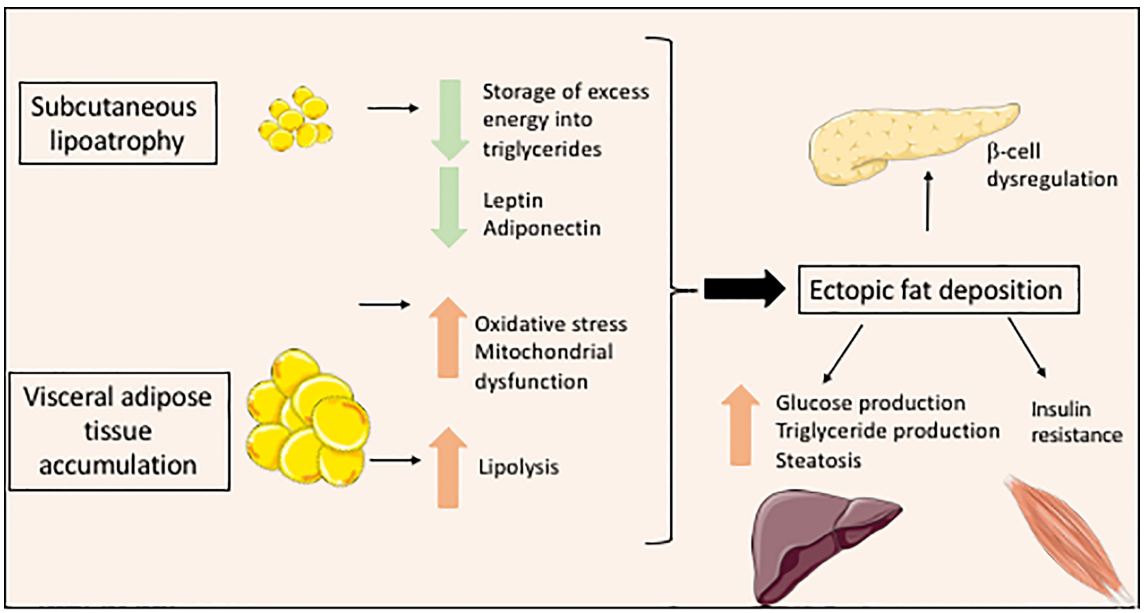

FIGURE 2 | Metabolic consequences of lipodystrophy leading to cellular lipotoxicity.

fat, is involved in adiposity-related hypertension (39). Mitochondrial dysfunction and oxidative stress, which are frequently observed cellular consequences of lipodystrophy, also decrease cellular responses to insulin (40–42) (**Figure 2**).

MAIN CLINICAL FEATURES OF GENERALIZED AND PARTIAL LIPODYSTROPHIES

The diagnosis of lipodystrophy syndromes is based first and foremost on clinical examination. Since objective clinical measures are still lacking to document an abnormal development of subcutaneous fat, the clinical diagnosis of lipodystrophy is highly dependent on the clinician's self-experience. It can be particularly difficult in men affected with partial lipodystrophy syndromes, in whom the diagnosis could be missed or substantially delayed. Indeed, the nosological framework is poorly defined between an android distribution of fat, commonly observed in the general population, which is a major risk factor for insulin resistance-related diseases (43), and the lipodystrophic phenotype. This is particularly striking for Type 1 Familial Partial Lipodystrophy (FPLD1), characterized by a central repartition of fat with lipoatrophy of limbs and severe insulin resistance, which is probably of polygenic origin (19). However, several clinical features are common to lipodystrophy syndromes, so that a distinctive clinical picture may be identified.

Lipoatrophy

The identification of generalized or segmental lipoatrophy, in the absence of malnutrition, is a major step for diagnosis.

In generalized forms of lipodystrophy syndromes, lipoatrophy of the face is striking. The patient' facies is emaciated due to the absence of Bichat fat pads. Acromegaloid features, with protruding eyebrow arches, cheekbones, and lower jaw, and thick facial traits, are also observed, especially in congenital forms of lipoatrophy, but also in some partial forms of the disease (8, 44). These clinical signs are due to an increased visibility of bone structures in the absence of adipose tissue, and to the stimulation of growth factor pathways by excess insulin. Hands and feet may also be thickened. The lack of subcutaneous adipose tissue also increases the visibility of muscles (athletic appearance) and of veins (pseudo-veinomegaly) in limbs, trunk and abdomen. In addition, it should be noted that, at least in some cases, the volume and mass of skeletal muscle are truly increased (45, 46) (**Figure 3A**). Ingrown toenails are possible consequences of severe lipoatrophy of feet. Hypomastia is common in women, secondary to loss of breast adipose tissue and/or to hyperandrogenism.

Fat Accumulation

In most forms of partial lipodystrophy syndromes, lipoatrophy mainly affects lower limbs and can coexist with areas of fat accumulation. Dunnigan syndrome (FPLD2), due to pathogenic variants in the *LMNA* gene encoding Type A lamins, is characterized by lipoatrophy of limbs and trunk with cushingoid features of face and neck, *i.e.* increased supraclavicular fat pads, double chin, buffalo hump, and with perineal accumulation of adipose tissue. The general morphotype is strikingly android, with a biacromial diameter greater than the bitrochanteric diameter (8, 47). Muscle hypertrophy, contrasting with the usual amyotrophy associated with hypercortisolism, may suggest the diagnosis of lipodystrophy syndrome (**Figure 3A**). Fat overgrowth may lead to

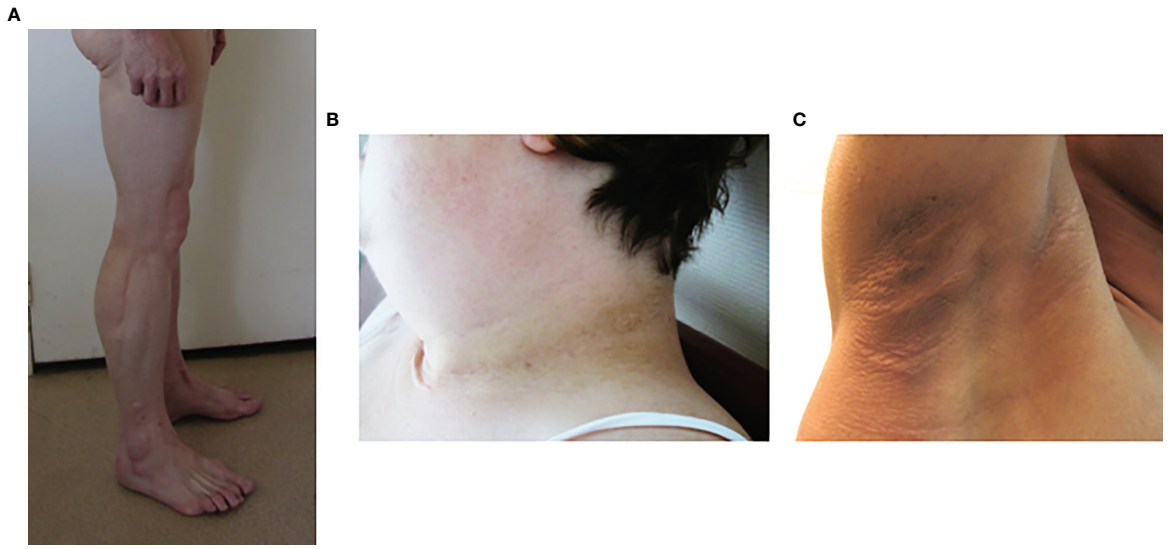

FIGURE 3 | Phenotypic features of lipodystrophy syndromes. **(A)** Muscular hypertrophy and lipoatrophy of limbs in Type 2 Familial Partial Lipodystrophy (Dunnigan syndrome). **(B, C)** Cervical and axillary *acanthosis nigricans* in patients with lipodystrophy due to *LMNA* **(B)** or *BSCL2* **(C)** pathogenic variants.

massive pseudo-lipomatous regions in upper body and proximal limb areas, contrasting with lipoatrophy of non-lipomatous regions, in specific genetic forms of partial lipodystrophy (48–55). On the contrary, in Barraquer-Simons acquired partial lipodystrophy syndrome, a progressive lipoatrophy develops in upper parts of the body (face, trunk and upper limbs), while adipose tissue accumulates in lower limbs.

Insulin Resistance-Associated Clinical Signs

Acanthosis nigricans, a brownish hyperkeratotic thickening of the skin, and acrochorda (skin tags), are very common clinical signs of insulin resistance in lipodystrophy syndromes (**Figures 3B, C**). These skin lesions are usually located in cervical, axillary and inguinal folds, but may be very extensive in some patients. Insulin resistance frequently leads to ovarian hyperandrogenism in women, with hirsutism, irregular menses and polycystic ovaries (47, 56, 57). Hepatomegaly, resulting from dysmetabolic liver steatosis, is common. Hypertriglyceridemia can be complicated, or even revealed, by acute pancreatitis (7, 58).

Cardiovascular Signs

High blood pressure is frequent, and can be very severe, in particular in partial lipodystrophies associated with pathogenic variants of *PPARG* encoding the adipogenic factor PPARγ (peroxisome proliferator-activated receptor gamma) (59–61). The risk of atherosclerosis is strongly increased, which could result from insulin resistance, diabetes, and hypertension (62), but also from genetic variants that directly target the vascular wall, as observed in *LMNA*-related lipodystrophies (40, 63, 64). In addition to non-specific diabetic cardiomyopathy and atherosclerosis, several cardiovascular complications can be observed in the different forms of lipodystrophies. Patients with congenital generalized lipoatrophy may suffer from hypertrophic cardiomyopathy, with or without hypertension, associated with ectopic cardiac fat and/or lipotoxicity (65, 66). Pathogenic variants in *LMNA* are responsible for lipodystrophy syndromes with early atherosclerosis and/or with dilated cardiomyopathy, rhythm and/or conduction disorders and/or extensive calcifications of cardiac valves (67–72). A regular cardiovascular screening, with cardiac ultrasound and stress test, and, if needed, coronary CT angiogram, 24-hour ECG monitoring, and/or cardiac MRI is required in most patients with lipodystrophy syndromes (7, 8).

Other Clinical Signs

Depending on their molecular cause, lipodystrophy syndrome can be associated with several other clinical signs. Moderate intellectual disability can be observed in type 2 Congenital Generalized Lipoatrophy (CGL) due to *BSCL2* pathogenic variants encoding seipin (11). Digestive signs are frequent in neonates or infants with CGL. In late infancy or adolescence, dysphagia can reveal megaesophagus, due to esophageal achalasia, in CGL due to pathogenic variants of *CAVIN1* or *CAV1*, encoding proteins involved in the formation of caveolae at the cell plasma membrane (73–77). Growth disorders, dysmorphic features with micrognathia, beaked nose, dental crowding, prominent eyes, dystrophic bones, and/or signs suggesting accelerated aging such as precocious whitening and/or loss of hair, sclerodermatous skin appearance, joint limitations, and/or muscle atrophy are hallmarks of progeroid lipodystrophies (67, 78, 79). Other signs such as precocious cataracts, trophic skin disorders, hypogonadism, predisposition to cancer can be associated in progeroid lipodystrophy syndromes due to defects in DNA repair (80, 81). Muscle functional defects are observed in some patients with lipodystrophy due to

pathogenic variants in *LMNA*, *CAVIN1*, or *PSMB8*, among other genes (67, 74, 82–85). Systemic inflammatory signs (fever, multiorgan inflammatory involvement affecting joints, skin, heart) are prominent features of lipodystrophies associated with rare autoinflammatory syndromes of genetic origin (86). The occurrence of some lipodystrophy syndromes in the context of panniculitis or autoimmune diseases, suggests that adipose tissue could be a target of immune tolerance disruption (87).

BIOLOGICAL CHARACTERISTICS OF LIPODYSTROPHY SYNDROMES

Insulin resistance, resulting from adipose tissue dysfunction and subsequent ectopic lipid deposition, is one of the hallmarks of lipodystrophy syndromes. High serum insulin levels, with normal or increased plasma glucose, are detected in the fasting state and/or during oral glucose tolerance test. Patients with diabetes display preserved or even increased C-peptide levels for a long time, and/or frequently require very high doses of insulin to achieve glucose control. Hypertriglyceridemia is also very frequent, and can reach very high values, associated with a high risk of acute pancreatitis. Low HDL-cholesterol is almost always present. Increased liver enzymes are also common features, due to liver steatosis or fibrosis. Biological signs of adipose dysfunction include decreased serum adiponectin, and either barely detectable leptin levels in generalized lipodystrophies, or lower leptin levels than predicted by body mass index in partial lipodystrophies. Creatine phosphokinase may be elevated, especially in patients with lipodystrophies and muscular dystrophy. Ovarian hyperandrogenism results from insulin resistance, with high levels of total and free testosterone and of Δ4-androstenedione, decreased sex-hormone binding globulin, and increased luteinizing hormone to follicle-stimulating hormone ratio (7, 8).

IMAGING INVESTIGATIONS

Although the diagnosis of lipodystrophy is mainly based on clinical examination, the objective measurement of fat mass by dual energy-ray absorptiometry (DEXA) is useful to determine the severity of lipoatrophy and, in partial forms of lipodystrophies, to document the altered segmental distribution of fat (**Figure 4A**). Abdominal ultrasound, computed tomodensitometry (CT), or magnetic resonance imagery (MRI) are required to search for liver steatosis and signs of cirrhosis (**Figure 4B**). In women, pelvic ultrasound may reveal ovaries of increased volume and/or with multiple follicles. Bone imaging can show precocious non-specific degenerative radiographic abnormalities in patients with familial partial lipodystrophies. Several bone lesions such as osteolysis, osteosclerosis or pseudo-osteopoikilosis are frequently present in generalized forms of lipodystrophy, and may lead to misdiagnosis (88, 89). Muscle

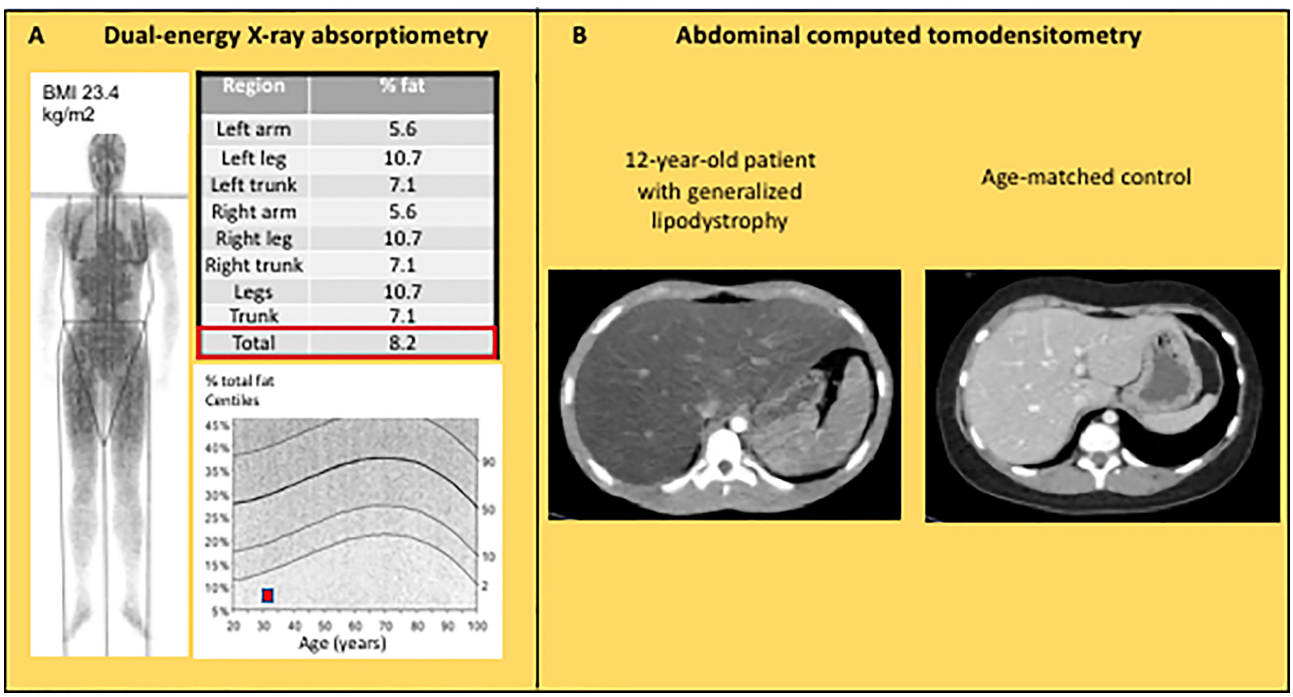

FIGURE 4 | Imaging features in lipodystrophy syndromes. **(A)** Dual energy-ray absorptiometry (DEXA) in a 31 year-old patient with CGL1, showing a major decrease in total and segmental fat mass. **(B)** Abdominal computed tomodensitometry in a 12 year-old patient with acquired generalized lipodystrophy showing homogeneous hepatomegaly with low attenuation of the parenchyma (Hounsfield units: -13), and absence of subcutaneous adipose tissue.

imaging (CT or MRI) may show muscular hypertrophy and/or fatty degeneration, with lack of subcutaneous adipose tissue. Imaging investigations may be completed by an electromyogram to search for neuropathic and/or myopathic signs.

LIPODYSTROPHY AND AGEING

Remodeling of body fat occurs during physiological ageing, with decrease in subcutaneous gluteofemoral adipose tissue, increase in visceral fat depots and ectopic deposition of lipids. Together with pro-inflammatory changes in adipose tissue, these lipodystrophy-like features contribute to age-related insulin resistance (90).

Accelerated ageing is probably one of the most important pathophysiological mechanisms of primary lipodystrophies. Arguments in favor of this hypothesis first came from studies of *LMNA*-associated diseases. *LMNA* encodes Type A lamins, nuclear proteins that interact with chromatin and regulate several nuclear functions including epigenetic cell developmental programs (91) (**Figure 1**). *LMNA* pathogenic variants cause several different laminopathies including muscular dystrophy, cardiomyopathy, neuropathy, lipodystrophy, and syndromes of accelerated aging (progeria and progeroid syndromes). Both typical FPLD2/Dunnigan syndrome, characterized by partial lipodystrophy and insulin resistance-related complications, and the extremely severe Hutchinson-Gilford accelerated ageing syndrome with generalized lipoatrophy are due to *LMNA* variants. In addition to lipodystrophy, several other clinical features, although of very different severity, are part of the clinical spectrum of both diseases. This is the case for early atherosclerosis, leading to frequent cardiovascular events before the age of 50 in patients with FPLD2, and to death at a mean age of 15 in Hutchinson-Gilford progeria (63, 92). In Hutchinson-Gilford progeria, but also in FPLD2, early atherosclerosis is due not only to metabolic risk factors, but also to direct pro-senescent effects of *LMNA* pathogenic variants on endothelial and vascular smooth muscle cells (40, 63, 93, 94). Clinical features of accelerated ageing are observed in patients with complex progeroid forms of *LMNA*-linked lipodystrophies, with a large continuum of severity between Dunnigan syndrome and Hutchinson-Gilford progeria (67, 71). At the cellular level, *LMNA* pathogenic variants impair the fate of several mesodermal lineages, such as endothelial vascular cells (64), myoblasts (95), cardiomyocytes (96), and adipocytes (97–101), and are involved in several signaling pathways which accelerate aging processes (102, 103).

The relationships between lipodystrophy and increased cellular senescence were further demonstrated by the identification of several pathogenic variants in genes involved in DNA repair as molecular causes of lipodystrophy syndromes (**Table 1** and **Figure 1**). As discussed above, lipodystrophies due to genetic alterations in DNA repair are clinically expressed as progeroid syndromes, with lipodystrophy, insulin resistance and related metabolic alterations, and signs of premature ageing. However, additional clinical features, specific to each different genetic subtype, may be part of the phenotype. Among others, cataracts, which are not part of the phenotype of *LMNA*-associated progeroid syndromes, are a typical feature of Werner progeria syndrome (81) and MDPL (Mandibular hypoplasia, Deafness, Progeroid features, and Lipodystrophy) syndrome (80, 104, 105). These diseases are due, respectively, to biallelic pathogenic variants in *WRN* encoding the WRN DNA helicase and exonuclease, and to heterozygous loss-of-function variants in *POLD1*, encoding a catalytic subunit of DNA polymerase δ, both enzymes playing a major role in maintaining genome stability. Lipodystrophy is associated with a predisposition to cancer in Werner syndrome, as well as in Bloom syndrome, due to mutations in *BLM* encoding a DNA helicase, or in ataxia-telangiectasia with altered DNA synthesis and excision-repair pathways (106). Cultured fibroblasts from patients with Werner or MDPL syndromes present signs of premature senescence (81, 107). Importantly, premature senescence was shown to impair adipogenesis in human pluripotent stem cells lacking either WRN or BLM helicases (108). Several other DNA replication/repair-associated lipodystrophies have been described (7, 78, 109, 110), frequently associated with short stature, hypogonadism, and trophic skin disorders, among other progeroid signs.

Premature senescence and oxidative stress, directly resulting from defects in genes involved in cellular ageing and/or genome stability, or from consequences of other genetic defects impacting adipocytes (79), are thus probably important pathophysiological determinants of lipodystrophies.

LIPODYSTROPHY AND IMMUNO-INFLAMMATORY DISEASES

Lipodystrophy syndromes can develop during the course of systemic immune and/or inflammatory diseases, suggesting that adipose tissue dysfunction could result from auto-immune and/or inflammatory damages. This is the case in CANDLE (Chronic Atypical Neutrophilic Dermatosis with Lipodystrophy and Elevated temperature) syndrome and related auto-inflammatory genetic diseases, due to genetic defects in immunoproteasome subunits (86, 111). Dysregulation of the interferon pathway is a key pathophysiological link between the molecular causes of these diseases and their clinical expression (112). Lipodystrophy with metabolic complications is also part of the phenotype of several autoimmune diseases. Generalized lipodystrophy with severe hyperinsulinemia and leptin deficiency has been described in a child with APECED (Autoimmune PolyEndocrinopathy-Candidiasis-Ectodermal Dystrophy) due to pathogenic variant in *AIRE* resulting in the disruption of immune tolerance (113). The term "acquired lipodystrophy", which designates lipodystrophy syndromes without any known genetic cause, underlies several auto-immune diseases with adipose tissue involvement (114–116). Autoantibodies directed against perilipin-1, an important lipid droplet protein which regulates lipolysis, could be involved in adipocyte dysfunction (87). Barraquer-Simons partial lipodystrophy syndrome, which predominantly affects women,

is associated in one third of the cases with decreased C3 and/or C4 complement factors, and with membranoproliferative glomerulopathy due to C3 nephritic autoantibodies (117, 118).

Recently, lipodystrophy syndromes have been reported during the course of targeted cancer therapy using immune checkpoint inhibitors (119–122). These agents, by releasing nonspecific immunosuppressive pathways, are known to lead to multiorgan auto-immune related adverse events. Inflammatory features and infiltration of adipose tissue with $CD3^+$ and/or cytotoxic $CD8^+$ lymphocytes have been identified in anti-PD1-related lipodystrophy syndromes, strongly suggesting that disrupted immune tolerance to adipocyte self-antigens could be the cause of lipodystrophy (119–122).

To note, HIV-related lipodystrophies which are reviewed elsewhere (4), also result from altered adipocyte differentiation and inflammatory dysregulation (123).

LIPODYSTROPHY AND LIPOMATOSIS: SOME SHARED PATHOPHYSIOLOGICAL MECHANISMS?

In specific genetic forms of partial lipodystrophy, due to *MFN2* or *LIPE* biallelic pathogenic variants, fat overgrowth may lead to massive pseudo-lipomatous regions in upper body and proximal limb areas, leading to the diagnosis of multiple symmetric lipomatosis. However, recent studies have shown that fat accumulates in non-encapsulated pseudo-lipomas, which differ from the organization of typical lipomas, and that patients also present with lipoatrophy of non-pseudo-lipomatous region and with lipodystrophy-related metabolic complications. Therefore, multiple symmetric lipomatosis could be, at least in *MFN2* and *LIPE*-related forms, an exacerbated form of partial lipodystrophy (49–55).

MFN2-associated lipodystrophy is a mitochondrial disease due to biallelic pathogenic variants including a specific *MFN2* p.Arg707Trp substitution. *MFN2* encodes mitofusin-2, a transmembrane protein of the outer mitochondrial membrane whose oligomerization drives mitochondrial fusion (124) (**Figure 1**). Lipoatrophy, low serum leptin and adiponectin levels, as well as adipose tissue mitochondrial defects, oxidative stress and increased expression of some thermogenic markers, provide evidence of adipose tissue dysfunction in patients with *MFN2*-associated lipodystrophy (52, 54). Apart from lipodystrophy/pseudo-lipomatosis, patients may present with other clinical signs of mitochondrial involvement, including Charcot-Marie-Tooth peripheral neuropathy.

LIPE biallelic pathogenic variants may also lead to pseudo-lipomatous forms of partial lipodystrophy (49, 50, 53, 55). *LIPE* encodes the key lipolytic enzyme hormone-sensitive lipase (HSL) (**Figure 1**), and *LIPE* pathogenic variants leading to lipodystrophy act through loss-of-function mechanisms. Functional studies using adipose stem cells have shown that defective lipolysis and impaired adipocyte differentiation, but also mitochondrial dysfunction, could contribute to pathophysiological mechanisms in *LIPE*-related lipodystrophy syndrome (55). Importantly, although the metabolic phenotype and potential lipodystrophy signs were not systematically investigated, some patients carrying mtDNA mutations responsible for the myoclonic epilepsy and ragged red fibers (MERRF) syndrome were also reported with multiple symmetric lipomatosis (125). Mitochondrial defects have also been described in the most frequent form of multiple symmetric lipomatosis, associated with excessive alcohol consumption (126).

Therefore, mitochondrial alterations could lead to both pseudo-lipomatous and/or lipodystrophic diseases. Whether other mitochondriopathies induce lipodystrophic diseases in humans, as shown in mice, requires further investigations (127).

METRELEPTIN TREATMENT OF METABOLIC COMPLICATIONS ASSOCIATED WITH LIPODYSTROPHY SYNDROMES

Lipodystrophy syndromes are multi-tissue diseases, which require a multidisciplinary management. Regarding metabolic alterations, dietary measures and physical activity are very important therapeutic tools. Indeed, avoiding a positive energy balance leading to ectopic lipid infiltration is a major objective to prevent and/or treat metabolic alterations in the context of adipose tissue failure (7). To date, no medication has proven to be effective to cure lipoatrophy. Metformin is frequently used as a first-line pharmacological drug to decrease insulin resistance. Statins are frequently prescribed to reduce the cardiovascular risk, and fenofibrate can be added in case of insufficient response on triglycerides. Medium chain fatty acid supplementation is used to reduce hypertriglyceridemia in children. Very limited data are available regarding the effects of antidiabetic drugs in patients with lipodystrophic diabetes. Very high doses of insulin therapy are frequently used, due to severe insulin resistance.

The orphan drug metreleptin, a recombinant leptin analog, is the only specific treatment for the metabolic complications of lipodystrophy syndromes. Metreleptin, administered by subcutaneous injection once daily, is used as a hormone replacement therapy in patients with leptin deficiency. Metreleptin therapy obtained a marketing authorization to treat the complications of leptin deficiency in patients with lipodystrophy in Japan in 2013, in USA in 2014 (for generalized forms), and in Europe in 2018. Although metreleptin was not studied in placebo-controlled trials in the context of rare diseases, and although it does not lead to the reconstitution of lacking adipose tissue, it was shown effective, as an adjunct to diet, on metabolic and hepatic parameters in generalized lipodystrophy syndromes (**Figure 5**). In patients with lipodystrophy, metreleptin replacement therapy prevents ectopic storage of lipids, by decreasing food intake due to central effects, and by directly increasing peripheral insulin sensitivity (128–131). Metreleptin therapy has been shown to increase insulin sensitivity and insulin secretion, to reduce hypertriglyceridemia, hyperglycemia, HbA1c and fatty liver disease, and to improve quality of life (3, 6, 7, 130, 132–134) (**Figure 5**). Other recent studies have shown that

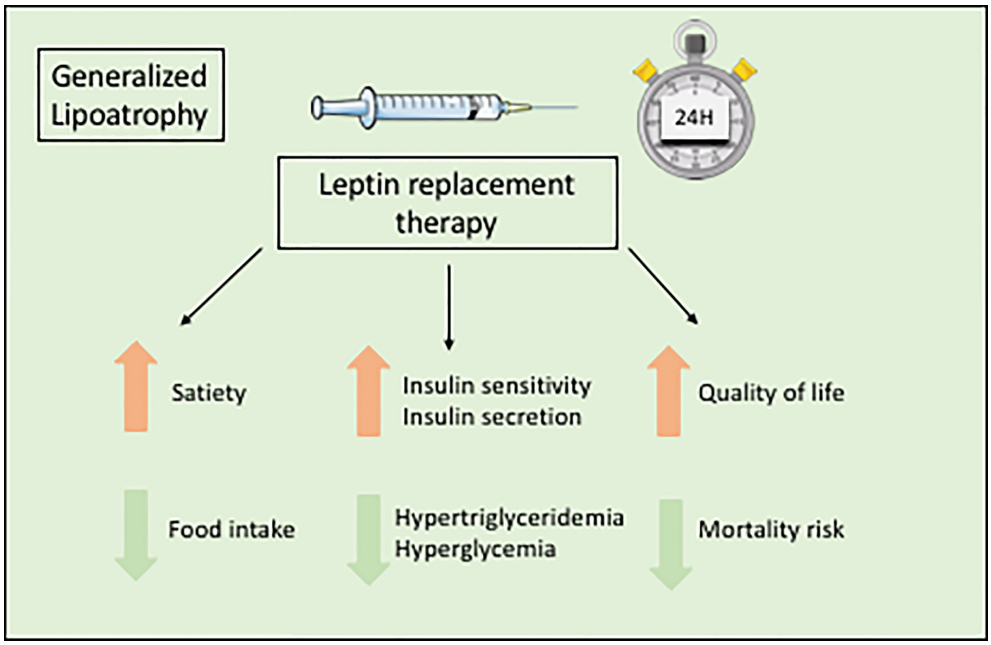

FIGURE 5 | Benefits of metreleptin replacement therapy in generalized lipodystrophies.

metreleptin could also improve cardiac hypertrophy by reducing lipotoxicity and glucose toxicity (135), and decrease mortality risk in patients with lipodystrophy (134). In accordance with the different severity of leptin deficiency, metreleptin therapy seems more efficient in generalized than in partial forms of lipodystrophy (136). In a recent *post-hoc* statistical analysis of two clinical trials conducted at NIH, HbA1c improved by a mean 2.16 percentage point after 12 months of treatment with metreleptin in patients with generalized forms of lipodystrophy (n=59), but only by a mean 0.61 percentage point in patients with partial lipodystrophy (n=36) (137). Therefore, in patients with partial lipodystrophies, metreleptin is recommended in selected patients, with severe hypoleptinemia, HbA1c > 8% and/or serum triglyceride > 500 mg/dL, for whom standard treatments have failed to achieve adequate metabolic control (7). Further studies are needed to determine specific predictive factors for metreleptin response in patients with partial lipodystrophies.

Metreleptin therapy is well tolerated in the majority of patients. The dose of metreleptin is adapted to metabolic responses and to tolerance, with particular attention to the extent of weight loss, which is an expected effect of the treatment but should be controlled. Common adverse effects mainly comprise localized skin reaction, pain at injection sites, and hypoglycemia when the decrease of other antidiabetic treatments is not sufficiently anticipated. Very rare cases of lymphoma have been reported in patients with autoimmune forms of lipodystrophy treated with metreleptin, without any established causal relationship with the treatment (138). Although they display only exceptionally neutralizing effects, circulating anti-leptin autoantibodies frequently develop under treatment. Anti-leptin autoantibodies interfere with enzyme-linked immunosorbent assays for serum leptin, complicating the correct interpretation of leptinemia in treated patients. Further studies are needed to identify other consequences of anti-leptin autoantibodies (6).

CONCLUSION

Lipodystrophy syndromes are rare and heterogeneous diseases. Their diagnosis is difficult and can be significantly delayed, since adipose tissue is not systematically investigated during clinical exam, and several symptoms are nonspecific (7). Most clinical forms of lipodystrophy remain genetically unexplained. Next generation sequencing technologies with exome or genome analysis will probably allow for discovering new causative genetic variants in the near future, and lead to a better understanding of the pathophysiology in these rare diseases. However, the interpretation of genetic variants is increasingly challenging (44). Closely associated genetic, clinical and fundamental research, as well as broad collaborations are needed to explore new pathophysiological determinants of lipodystrophy syndromes, and improve the care of affected patients.

AUTHOR CONTRIBUTIONS

JZ and CVi wrote the original draft. CVa, EC, MA, CS-L, EB, HM, BD, SJ, BF, and IJ reviewed and edited the manuscript and

the figures. All authors contributed to the article and approved the submitted version.

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GLOSSARY

Acromegaloid features	clinical signs typically associated with acromegaly, due to growth hormone overproduction, which may also be observed in insulin resistance syndromes, i.e. broadened extremities and coarsening of facial lines with widened and thickened nose, prominent cheekbones, and enlarged forehead
Android fat distribution/ morphotype	body fat distribution characterized by a predominant localization of adipose tissue in abdominal and upper thoracic regions
Autoinflammatory diseases	heterogeneous group of diseases characterized by recurrent inflammatory episodes with fever and increased inflammatory markers, due to a dysregulation of innate and/or adaptive immunity
Bichat fat pads	subcutaneous facial fat of the cheeks and temples
Cellular lipotoxicity	cellular dysfunction mediated by the accumulation of fatty acids derivatives
Cushingoid features	clinical signs typically associated with Cushing syndrome, due to corticosteroid overproduction, which may also be observed in some partial lipodystrophy syndromes, i.e. rounded face, doubled chin, supraclavicular, axillar and dorsocervical fat accumulation (buffalo hump)
Diabetic cardiomyopathy	myocardial dysfunction in the absence of overt clinical coronary artery disease or valvular disease observed in patients with diabetes mellitus
Liver steatosis	lipid accumulation in the liver which may lead to liver dysfunction, inflammation and fibrosis
Metabolic inflexibility	inability to adapt substrate oxidation to nutrient availability and hormone regulation – for example, in insulin resistance states, inability to switch from lipid to carbohydrate oxidation upon insulin stimulation
Multisystem diseases	disorders that affect several organs or tissues involved in specialized functions or in different physiological systems (i.e. cardiovascular system, endocrine system, central or peripheral nervous system, digestive system, immune system …)
Osteolysis	destruction of bone tissue
Osteosclerosis	localized or diffuse increased density of bone tissue
Progeroid syndromes	heterogeneous group of rare diseases characterized by clinical features of accelerated aging
Pseudo-lipomatous regions/pseudo-lipomas	unencapsulated masses of adipose tissue which can be clinically misdiagnosed as lipomas (encapsulated benign tumors of fatty tissue)
Pseudo-osteopoikilosis	numerous islands of osteosclerosis in the skeleton detected as spotted lesions on x-ray pictures
Segmental lipoatrophy	loss of adipose tissue involving a part of the body
Trophic skin disorders (observed in progeroid syndromes)	skin atrophy, dry and/or rigid skin with increased visibility of veins, changes in color and temperature, and/or impaired wound healing

frontiers in Endocrinology

REVIEW
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Pregnancy in Women With Monogenic Diabetes due to Pathogenic Variants of the Glucokinase Gene: Lessons and Challenges

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Heterozygous loss-of-function variants of the glucokinase (*GCK*) gene are responsible for a subtype of maturity-onset diabetes of the young (MODY). *GCK*-MODY is characterized by a mild hyperglycemia, mainly due to a higher blood glucose threshold for insulin secretion, and an up-regulated glucose counterregulation. *GCK*-MODY patients are asymptomatic, are not exposed to diabetes long-term complications, and do not require treatment. The diagnosis of *GCK*-MODY is made on the discovery of hyperglycemia by systematic screening, or by family screening. The situation is peculiar in *GCK*-MODY women during pregnancy for three reasons: 1. the degree of maternal hyperglycemia is sufficient to induce pregnancy adverse outcomes, as in pregestational or gestational diabetes; 2. the probability that a fetus inherits the maternal mutation is 50% and; 3. fetal insulin secretion is a major stimulus of fetal growth. Consequently, when the fetus has not inherited the maternal mutation, maternal hyperglycemia will trigger increased fetal insulin secretion and growth, with a high risk of macrosomia. By contrast, when the fetus has inherited the maternal mutation, its insulin secretion is set at the same threshold as the mother's, and no fetal growth excess will occur. Thus, treatment of maternal hyperglycemia is necessary only in the former situation, and will lead to a risk of fetal growth restriction in the latter. It has been recommended that the management of diabetes in GCK-MODY pregnant women should be guided by assessment of fetal growth by serial ultrasounds, and institution of insulin therapy when the abdominal circumference is ≥ 75th percentile, considered as a surrogate for the fetal genotype. This strategy has not been validated in women with in GCK-MODY. Recently, the feasibility of non-invasive fetal genotyping has been demonstrated, that will improve the care of these women. Several challenges persist, including the identification of women with *GCK*-MODY before or early in pregnancy, and the modalities of insulin therapy.

Yet, retrospective observational studies have shown that fetal genotype, not maternal treatment with insulin, is the main determinant of fetal growth and of the risk of macrosomia. Thus, further studies are needed to specify the management of *GCK*-MODY pregnant women during pregnancy.

Keywords: glucokinase, *GCK*-MODY, pregnancy, macrosomia, genotype, non-invasive fetal genotyping, insulin therapy

INTRODUCTION

Heterozygous pathogenic variants of the glucokinase (*GCK*) gene are associated with an autosomal dominant monogenic diabetes called *GCK*-maturity-onset-diabetes of the young (*GCK*-MODY). The *GCK*-MODY phenotype is restricted to a mild hyperglycemia that usually does not require any treatment. However, during pregnancy, the glucose levels of *GCK*-MODY mothers are high enough to potentially generate adverse outcomes similar to those observed in other forms of pregestational or gestational diabetes. The goal of the present review is to summarize current knowledge and challenges about this condition, its diagnosis, and its treatment during pregnancy. The literature was searched using the terms "glucokinase" or "GCK-MODY" or "MODY2" and "pregnancy", and all clinical publications were reviewed. Single case reports were not retained, unless they provided important information (e.g. the occurrence of congenital malformations).

DIABETES IN PREGNANCY

The relationships between diabetes and pregnancy are classically considered, according to the onset of diabetes before the beginning of pregnancy ("pregestational diabetes") or during the course of the pregnancy ("gestational diabetes") (1). Pregestational diabetes may be responsible for complications such as miscarriage, the occurrence of congenital malformations, and the subsequent increased risk of fetal or neonatal mortality. These complications are mainly related to the degree of hyperglycemia during the very first weeks of pregnancy (2). Pregestational as well as gestational diabetes may be responsible for many adverse issues for the mother, such as an increased frequency of hypertension, pre-eclampsia, pre-term delivery, and cesarean delivery; and for the baby, particularly macrosomia (i.e. excessive growth for gestational age), the associated risks of dystocia and neonatal hypoglycemia, and the consequences of prematurity (1). Moreover, maternal hyperglycemia might be responsible for the long-term occurrence of obesity and metabolic or cardiovascular diseases in the exposed offspring (3–6), although this remains a matter of debate (7).

The deleterious role of hyperglycemia during pregnancy has been further demonstrated by intervention studies in which "near normal" maternal blood glucose levels led to a decrease of adverse events (8, 9). These observations have led to a "glucocentric" approach of diabetes care in pregnancy, that is too restrictive (10), and other potentially modifiable risk factors have been identified, such as pre-pregnancy obesity, excessive weight gain during pregnancy, and gestational hypertension (11–13). Recent studies also showed that in women with gestational diabetes, beyond the degree of hyperglycemia, the respective roles of insulin deficiency and insulin resistance in the pathophysiology of mother's diabetes may have differential impacts on pregnancy adverse events (14, 15). Moreover, in addition to maternal blood glucose levels, the fetal genotype may strongly influence birthweight and the risk of macrosomia (16).

MONOGENIC DIABETES DUE TO GLUCOKINASE MOLECULAR ALTERATIONS

Monogenic diabetes (MgD) due to pathogenic alterations of the glucokinase gene (*GCK*), the first identified genetic subtype of MODY (17), called *GCK*-MODY (formerly MODY2), is among the most common MgD subtype (18), with an estimated prevalence of 0.1% in the general population (19). Its unique characteristics have allowed drawing important lessons, particularly on the role of maternal and fetal genetics in the consequences of diabetes during pregnancy.

The Genetics of *GCK*-MODY

Heterozygous inactivating variants in *GCK* are responsible for *GCK*-MODY. More than 900 *GCK* mutations have been reported (Human Gene Mutation Database, HGMD-2021-3*)*, including single-nucleotide variants (SNV) and rare exonic or gene deletions (20). The variants are distributed throughout the gene, with no mutational hotspots, and most are private. Pathogenic *GCK* variants lead to altered enzyme kinetics and more rarely to enzyme instability (20). No genotype/phenotype correlations have been reported (18, 21), with a similar phenotype in most patients, probably due to partial compensation of glucokinase activity by the wild-type allele (22). The molecular diagnosis of *GCK*-MODY in a proband relies on the search of SNV and large deletions, based on either analysis restricted to *GCK* (including sequencing and dosage analysis) if the proband's phenotype is highly suggestive of *GCK*-MODY, or the sequencing of a multigene panel including *GCK*. In all cases, determining whether an identified variant is disease-causing, a normal variation, or a variant of unknown significance is a key step in the diagnostic process reviewed in (23).

Pathophysiology of *GCK*-MODY

Glucokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate, the first and rate-limiting step of glucose metabolism in the pancreatic beta-cell, which regulates insulin secretion in proportion to glucose metabolism within the physiological range. Glucokinase is thus considered as the "glucose sensor" of the pancreas (24). In patients with *GCK*-MODY, the curve of insulin secretion in response to increasing glucose concentrations is shifted to the right, and the glucose threshold for insulin release is higher than in normal individuals (25). Thus, fasting hyperglycemia is one main metabolic alteration in *GCK*-MODY. In the liver, glucokinase catalyzes the first step of glucose storage by glycogen synthesis, and patients with *GCK*-MODY harbor a decrease of hepatic glycogen synthesis and a relative increase of neoglucogenesis, which both participate to increased post-prandial glucose levels (26). *GCK* is also expressed in the pancreatic alpha-cells and in hypothalamus, and counterregulation to hypoglycemia also occurs at higher blood glucose levels in *GCK*-MODY patients (27, 28).

Insulin sensitivity is usually considered to be unaffected in *GCK*-MODY patients. However, studies showed that it was lower in patients with *GCK* mutations as compared to their non-affected relatives, and was negatively associated with a mild deterioration of glucose tolerance, consistent with a deleterious effect of chronic hyperglycemia (29–31). Conversely, a longitudinal study suggested that the deterioration of glucose tolerance observed in some *GCK*-MODY patients was due to a decrease in insulin sensitivity, which could be related to aging, weight gain, and/or polygenic susceptibility (32). Whether this may also occur during pregnancy, a known situation of decreased insulin sensitivity, is not known. To our knowledge, no longitudinal study assessed whether glucose tolerance deteriorates in women with *GCK*-MODY during pregnancy, and improves after delivery. However, the fasting and 2 hours after a 75 g oral glucose tolerance test (OGTT) blood glucose values measured in 44 pregnant women with *GCK*-MODY were in the same range as those of non-pregnant *GCK*-MODY patients (19).

The Main Characteristics of *GCK*-MODY Patients

As compared to Type 1, Type 2 and other monogenic diabetes, *GCK*-MODY has unique characteristics reviewed in (18). The main feature observed in *GCK*-MODY patients is a mild fasting hyperglycemia, typically in a narrow range (5.4-8.3 mmol/L), with an increase 2 hours after a 75 g OGTT usually < 3.0 mmol/L (21, 33, 34). HbA_{1c} values are comprised between 38 and 60 mmol/mol (5.6-7.6%), and allow good discrimination of the patients from non-carriers relatives, and from patients with Type 1 or Type 2 diabetes (35). Hyperglycemia is present from birth and blood glucose levels increase mildly with age, as observed in non-diabetic individuals, albeit at a higher level (33, 35, 36). The penetrance of this phenotype is complete, all carriers of a *GCK* defect being hyperglycemic, generally at the same level (33).

Patients with *GCK*-MODY are clinically asymptomatic and, in the absence of other risk factors, micro- and macrovascular complications are rare, except for a mild non-clinically significant retinopathy, even in patients with long-standing hyperglycemia (36).

Consequently, outside pregnancy, no treatment of hyperglycemia is warranted in *GCK*-MODY patients. Moreover, treatments of hyperglycemia are not effective in these patients. In prospective studies, 20-50% of *GCK*-MODY patients were treated with oral hypoglycemic agents or insulin before the diagnosis was made. HbA_{1c} values were very similar in treated and untreated patients, and were not affected by treatment withdrawal (37–39). It is likely that the up-regulated counterregulation of *GCK*-MODY patients will prevent strict normalization of blood glucose values (27, 28).

RISKS ASSOCIATED WITH PREGNANCY IN WOMEN WITH *GCK*-MODY

The Rate of Miscarriage Would Be Expected to Be Increased Given the Blood Glucose and HbA_{1c} Levels Observed in *GCK*-MODY Women

It has been reported to be increased compared to that of the general population (33% of 56 pregnancies) in one study (40), but similar to the general population rate in a further study by the same group on a larger population (17% of 119 pregnancies) (41), and in an additional independent study (19% of 128 pregnancies) (42).

The Risk for Congenital Malformations Has Not Been Systematically Assessed

Blood glucose levels of *GCK*-MODY patients are compatible with a slightly increased risk, i.e. a 30% risk increase per 1% (11 mmol/mol) increase in HbA_{1c} above 6.3% (45 mmol/mol), if one refers to data obtained in women with pregestational diabetes (2). One case of caudal regression syndrome was reported, and the potential benefit of systematic peri-conceptual folic acid supplementation in women with known *GCK*-MODY has been suggested (as actually recommended in the general population) (43). A pulmonary valve stenosis was reported in a child born to a *GCK*-MODY mother, whose early pregnancy HbA_{1c} was 6.5% (48 mmol/mol) (44). Also, 4 congenital malformations were reported among 99 offspring born to mothers with *GCK*-MODY, which could be higher than in the general population, but no further information was available (41). Thus, in *GCK*-MODY women with a pre-conceptual HbA_{1c} > 6.3% (45 mmol/mol), insulin therapy could be recommended to prevent the risk of congenital malformations.

Unaffected Offspring of Mothers With *GCK*-MODY Are at High Risk of Macrosomia

Blood glucose and HbA_{1c} values observed in patients with *GCK*-MODY confer a high risk of macrosomia (45). In women with pregestational diabetes, the probability of large for gestational age (LGA, defined by a birthweight > 90^{th} percentile) offspring increases linearly with third trimester HbA_{1c} values above 36.6 mol/mol (5.5%) and is almost maximal (65% risk) for a 53 mmol/mol

(7.0%) value (12). Even in offspring born to women with mild gestational diabetes, the risk of LGA increased linearly with increasing blood glucose levels up to 5.8 mmol/L fasting and to 11.1 mmol/L at 2 hours of a 75 g OGTT, and was associated with a parallel increase in cord blood C-peptide levels (a measure of insulin secretion by the fetus) (46).

Given the autosomal transmission of *GCK*-MODY, at each pregnancy the probability that a fetus will inherit the maternal *GCK* mutation is 50%. In this context, fetal growth will dependent both on the degree of maternal hyperglycemia, and on the ability of the fetus to increase insulin secretion in response to hyperglycemia, i.e., its *GCK* genotype. Indeed, the seminal report by A.T. Hattersley et al. on *GCK*-MODY families clearly showed that non-affected offspring born to affected mothers had a 600 g higher birth weight than affected offspring born to affected mothers and than non-affected offspring born to non-affected mothers (47). According to the Pedersen hypothesis (48), this suggested that accelerated fetal growth was induced by increased fetal insulin secretion by non-affected fetuses in response to maternal hyperglycemia, and that fetal growth was normal in affected fetuses since their insulin secretion was set at the same level as their mother's. Observational studies have consistently confirmed this report (41, 42, 49–52), and showed that the frequency of macrosomia (birthweight > 4000 g) and/or of LGA offspring were high (33-65%) in the first situation, as compared to the latter (4-13%, i.e. the expected rate of LGA in the general population) (**Table 1**). However, in one series 5/15 affected offspring born to affected mothers were macrosomic. These five infants were born to 4 mothers who had had 10 other pregnancies, and delivered 8 macrosomic infants. This suggested that confounding factors, yet unidentified, may have played a role in the occurrence of excessive fetal growth (42). Of note, in the offspring of an unaffected mother inheriting a *GCK* mutation from their father, birth weight was reduced by 500 g, compared to controls, confirming the central role of fetal insulin secretion in the fetal growth (47).

Macrosomia can be associated with increased frequencies of many perinatal adverse outcomes, including shoulder dystocia, fetal distress, perineal tears, induced preterm delivery and Cesarean delivery (CS), neonatal hypoglycemia, and neonatal respiratory distress. These complications have been reported in the context of pregestational diabetes, as well as in mild gestational diabetes, where blood glucose levels are quite similar to that observed in *GCK*-MODY (46). Shoulder dystocia was reported in 4/15 macrosomic non-affected babies born to *GCK*-MODY mothers (50). Other adverse outcomes, mainly emergency or planned CS due to macrosomia, have also been reported (42). However, the rates of these complications have not been systematically assessed in unaffected offspring born to affected mothers, compared to affected offspring.

Unaffected Offspring of Mothers With *GCK*-MODY Do Not Exhibit Clinical or Metabolic Abnormalities in the Long Term

In various situations, fetal exposure to maternal diabetes has been associated with long-term deleterious effects, particularly defects in glucose-stimulated insulin secretion and hyperglycemia. This has been shown in offspring of mothers with type 2 diabetes (53–55), with monogenic diabetes associated with hepatocyte nuclear factor 1 alpha (56), but also with type 1 diabetes (57). As regards *GCK*-MODY, two studies demonstrated no obvious long-term effects in offspring of *GCK*-MODY mothers. In the first one, 42 adult non-mutation carriers born to *GCK*-MODY mothers were compared to 39 non-mutation carriers born to unaffected mothers, at a median age of 42 and 36 years, respectively. No differences were observed in fasting and post-load (75 g OGTT) glucose values, insulin secretion and insulin sensitivity indexes, body mass index (BMI), blood pressure and lipid profiles (49). In the second study,

TABLE 1 | Birthweight percentiles, frequency of large for gestational age newborns and gestational age at delivery according to fetal genotype and treatment of diabetes in GCK-MODY mothers.

Reference N°	Effect of fetal genotype		Effect of fetal genotype and maternal treatment			
	GCK –	GCK +	GCK – diet	GCK – insulin	GCK + diet	GCK + insulin
(50)	Pc = 85 ± 21 (38)	Pc = 47 ± 31 (44)*	Pc = 86 ± 22 (19)	Pc = 84 ± 21 (19)	Pc = 51 ± 30 (30)	Pc = 39 ± 33 (14)
	LGA = 21/38 (55%)	LGA = 4/44 (9%)*	–	–	–	–
	T = 38.1 ± 1.7	T = 38.7 ± 2.6†	T = 38.9 ± 1.7	T = 37.3 ± 1.1‡	T = 39.1 ± 2.7	T = 37.8 ± 2.0‡
(51)	–	–	–	–	–	–
	LGA = 9/22 (41%)	LGA = 4/45 (9%)*	–	–	–	–
	T = 38.7 ± 2.7	T = 39.3 ± 2.3	–	–	–	–
(52)	Pc = 75 ± 27 (12)	Pc = 41 ± 31 (28)*	Pc = 86 ± 10 (8)	Pc = 53 ± 37 (4)‡	Pc = 41 ± 31 (19)	Pc = 40 ± 31 (9)
	LGA = 4/12 (33%)	LGA = 1/28 (4%)*	–	–	–	–
	T = 39.3 ± 1.0	T = 38.4 ± 2.3	T = 39.3	T = 39.4	T = 38.8	T 37.6
(42)	–	–	Pc = 90 ± 8 (3)	Pc = 84 ± 22 (9)	Pc = 58 ± 33 (15)	Pc = 34 ± 27 (8)
	–	–	–	–	–	–
	–	–	T = 36	T = 37	T = 40.4	T = 38.0‡
(41)	–	–	Pc = 69 ± 34 (12)	Pc = 92 ± 18 (11)	Pc = 50 ± 28 (28)	Pc = 64 ± 35 (11)
	LGA = 15/23 (65%)	LGA = 5/39 (13%)*	LGA = 6/12 (50%)	LGA = 9/11 (82%)	LGA = 1/28 (4%)	LGA = 4/11 (36%)
	–	–	T = 39.5 ± 1.5	T = 38.3 ± 1.0‡	T = 39.6 ± 1.0	T = 38.7 ± 1.4‡

*Data are: 1st line: mean ± SD of birth weight percentiles (Pc) with numbers of cases into parentheses; 2nd line: numbers of large for gestational age (LGA) newborns/total numbers of newborns, with percentages into parentheses; and 3rd line: mean term (T) at delivery (weeks). LGA was defined as a corrected birthweight > 90th percentile. *significantly lower than in GCK - babies; †significantly higher than in GCK - babies; ‡significantly lower than in diet treated babies.*

15 unaffected offspring of *GCK*-MODY mothers were compared to 14 unaffected offspring of *GCK*-MODY fathers. Although the former were on average 720 g heavier at birth, they did not display any alteration of glucose tolerance, insulin secretion, BMI, percentage of body fat mass, and blood pressure, at ~ 36-39 years of age (58). These observations suggest that fetal exposure to maternal hyperglycemia ("metabolic imprinting") is not always sufficient to induce long-term metabolic abnormalities in the offspring.

THE DIAGNOSIS OF *GCK*-MODY BEFORE AND DURING THE COURSE OF PREGNANCY

Outside the Pregnancy, the Diagnosis of *GCK*-MODY May Be Raised on the Discovery of Hyperglycemia

In young and lean individuals, type 1 diabetes should be excluded by the absence of diabetes-related autoantibodies (59). The criteria in favor of *GCK*-MODY include the mild degree of hyperglycemia (5.5-8.0 mmol/L), a 2 hr increment < 4.6 mmol/L on a 75 g OGTT, and a family history of hyperglycemia, including gestational diabetes, suggesting an autosomal dominant inheritance (34). A young age and a normal BMI at the time of first recognition of hyperglycemia are also in favor of the diagnosis, since they are not typical of Type 2 diabetes. The family history of hyperglycemia may be unrecognized, or even absent in the rare cases of *de novo* occurrence of a *GCK* mutation. In all cases, the suspicion of *GCK*-MODY in a proband should lead to measure fasting blood glucose in the parents (34). It is also important to systematically screen fasting blood glucose in relatives of a patient with *GCK*-MODY, since this will identify women of child-bearing age who should be genetically tested. Nevertheless, it has been estimated that almost all *GCK*-MODY cases are not diagnosed (19). Thus, it can be anticipated that in the majority of women with *GCK*-MODY hyperglycemia will be first detected during pregnancy, owing to screening for gestational diabetes.

Screening for Gestational Diabetes Is an Opportunity to Diagnose *GCK*-MODY

For decades, the diagnosis of gestational diabetes has been a matter of debate as regards the women who should be screened, the optimal term of pregnancy to perform screening, and which test should be used (60). Some have suggested to screen all women when planning pregnancy, or at the latest at first prenatal contact, by measuring fasting plasma glucose (61, 62), which could be a good opportunity for *GCK*-MODY diagnosis. However, it is generally recommended to screen women with risk factors (age ≥ 35 years, pre-pregnancy BMI ≥ 25 kg/m², first-degree relative with diabetes, history of gestational diabetes, or of delivery of a macrosomic neonate), as soon as possible during pregnancy, and all women at 24 weeks of gestation (60). Among the risk factors for gestational diabetes, some may be present in women with *GCK*-MODY.

The reported prevalence of *GCK*-MODY among women with a diagnosis of gestational diabetes is typically 0.5-2%, but varies considerably (from 0 to 80%) according to the criteria used for genetic screening reviewed in (63, 64). Using stringent criteria to select women for genetic screening will increase the diagnosis rate, but at the cost of a lower sensitivity. New pregnancy-specific screening criteria (NSC) have been defined in a population-based study of women with gestational diabetes, of whom ~ 1% had a confirmed *GCK*-MODY (19). They include an antepartum fasting blood glucose ≥ 5.5 mmol/L (99 mg/dl) and a pre-pregnancy BMI < 25 kg/m². In the studied white European population, these criteria had a 68% sensitivity and a 99% specificity for the diagnosis of *GCK*-MODY. Using these criteria, the number of women needed to test to find one *GCK*-MODY case was 2.7. Decreasing or increasing the BMI threshold decreased or increased sensitivity, respectively, with no major effect on specificity (19). A multiethnic Australian study confirmed that these criteria performed well in women of Anglo-Celtic origin, but not in those of Asian or Indian origin (65). Among Danish women with diet-treated gestational diabetes, 2% had *GCK*-MODY, but a BMI < 25 kg/m² was not discriminant in this study (66). Thus, more multiethnic studies in women with gestational diabetes are needed to refine the criteria for genetic screening, and the diagnosis of *GCK*-MODY in early pregnancy is currently an unsolved challenge.

MANAGEMENT OF PREGNANCY IN WOMEN WITH *GCK*-MODY

Current Recommendations on the Treatment of *GCK*-MODY During Pregnancy

These recommendations mainly apply to the use of insulin therapy to prevent macrosomia and its potential consequences reviewed in (67). As mentioned, when the fetus has not inherited the maternal mutation, the risk of macrosomia is high, and normalization of maternal blood glucose levels is necessary. By contrast, when the fetus has inherited the maternal mutation, treatment of maternal hyperglycemia should be avoided because of the risk of fetal growth restriction due to a decrease of fetal blood glucose values under the insulin secretion threshold (68, 69). Thus, knowing the fetal genotype will determine whether the maternal hyperglycemia should be treated. The proof of concept of this approach was obtained in two *GCK*-MODY women in whom chorionic villous sampling, performed for other reasons, showed the presence of the mutation in the fetuses. No treatment of diabetes was initiated and the women delivered normal-weight babies (70, 71).

However, invasive prenatal diagnosis conveys risks and is not appropriate for a benign condition, and the fetal genotype is unknown in almost all cases. Thus, so far it has been suggested that the course of fetal growth, monitored by serial ultrasounds (US), may be used as a surrogate for the fetal genotype, and for the need for treatment of maternal hyperglycemia (18).

This strategy is adapted from studies performed in women with "common" gestational diabetes. All offspring of women

with gestational diabetes are not at risk for macrosomia, and "too tight" control of maternal hyperglycemia may lead to an increased risk of intrauterine growth retardation (72). Thus, several randomized trials were performed in women with gestational diabetes, comparing the initiation of insulin therapy based on maternal blood glucose values, or on an accelerated fetal growth, defined by an abdominal circumference (AC) ≥ 70^{th}-75^{th} percentile on US (73–76). US allowed to identify the infants at low risk of macrosomia, and to avoid insulin therapy in their mothers, with no increase of pregnancy adverse outcomes, particularly no increase of LGA, nor of small for gestational age offspring (77). Of note, insulin therapy was also introduced in the US groups when maternal blood glucose values exceeded safety levels, 6.7 mmol/L (120 mg/dl) fasting or 11.1 mmol/L (200 mg/dl) post-meal, i.e., much above the usually recommended targets in women with gestational diabetes. However, when insulin therapy had to be initiated in at risk pregnancies, strict blood glucose targets (4.4 and 6.1 mmol/L, 80 and 110 mg/dl, fasting and after meals, respectively) were set to reduce the risk of macrosomia (77). Although this approach has been validated in a real life setting (78), its benefits have been questioned in a recent review (79) and it is not part of the current guidelines on the management of gestational diabetes (1).

The same approach has been recommended in pregnant women with *GCK*-MODY (68), including an US every two weeks, starting from 26 weeks of gestation, with the AC ≥ 75^{th} pc threshold for initiation of insulin therapy (**Figure 1**). In women treated with insulin, it is recommended that delivery should be induced at 38 weeks (18). Several potential pitfalls can be raised, including the limited accuracy of fetal US to predict the risk of macrosomia, due to intra-and inter-observer variability of AC measurement, the restricted availability of high-quality US, and the relatively late identification of the risk of macrosomia, at a time when intervention might be less efficient (80). To date, this strategy has not been validated in *GCK*-MODY and should be considered as expert opinion.

A prospective study has been recently completed in women with *GCK*-MODY, comparing the two strategies, i.e., institution of insulin therapy according to blood glucose values or to fetal growth (ClinicalTrials-NCT02556840). Analysis is ongoing and will hopefully show whether the second approach is safe, and to what extent insulin therapy is efficient to control maternal blood glucose levels and fetal growth in unaffected offspring.

Non-Invasive Prenatal Testing

The presence of fetal DNA in maternal plasma from the early first trimester of gestation has allowed the development of non-invasive prenatal testing (NIPT) for single-gene diseases (81). However, NIPT for maternally inherited variants presents technological and analytical challenges because only a small proportion (5% to 20%) of the total cell-free DNA in maternal plasma is derived from the fetus during early pregnancy. Two major methods for NIPT have been developed. One uses droplet digital PCR to quantify reference and alternate alleles and to estimate the allelic balance of the mutation itself. The second consists in the identification of at-risk maternal haplotype at a specific locus using high-throughput DNA sequencing technologies and the determination of the maternal haplotype

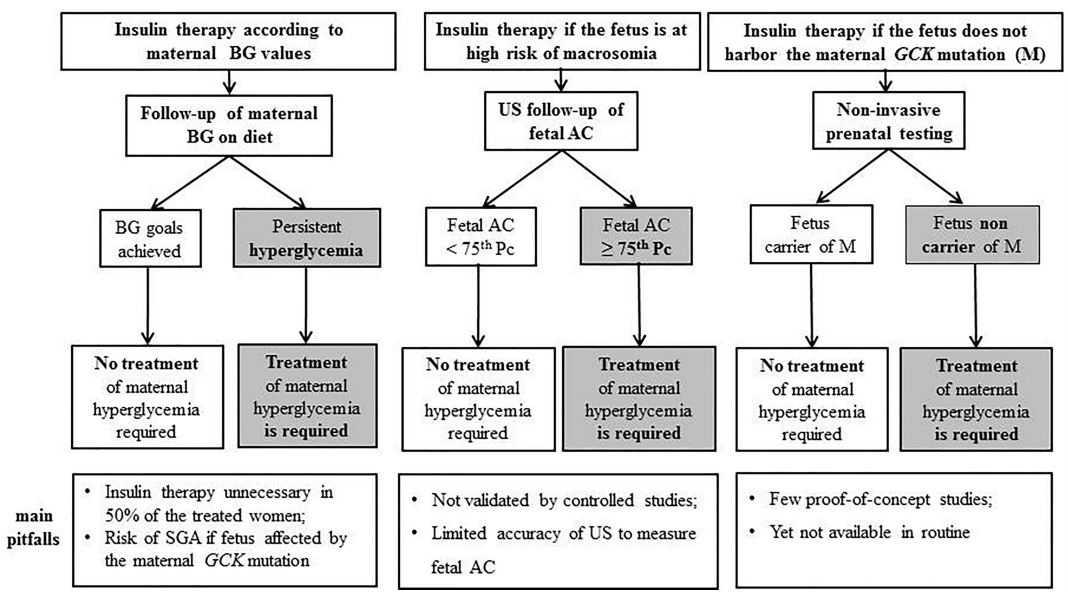

FIGURE 1 | Suggested algorithms to initiate insulin therapy in pregnant women with *GCK*-MODY. The left part of the figure describes the approach based, as in "common" gestational diabetes, on maternal blood glucose (BG) values. The middle part illustrates current recommendations, based on the serial measurement of fetal abdominal circumference (AC) by ultrasounds (US), and initiation of insulin therapy when AC is ≥ 75^{th} percentile (Pc), which suggests the absence of the maternal *GCK* mutation in the fetus and a risk of macrosomia. In the right part, initiation of insulin therapy will be based on the absence of the maternal mutation (M) in the fetus, diagnosed by non-invasive prenatal testing. The bottom part of the figure indicates the main pitfalls of each strategy. SGA, small for gestational age.

transmitted to the fetus, based on relative haplotype dosage estimation. Both methods have recently been successfully performed in pregnant women with *GCK*-MODY as proof-of-concept studies (82–84). In these studies, NIPT could effectively be performed from 12 weeks of gestation, with a current 3-5 week time to results, and both high sensitivity (87%) and specificity (100%) (82). The possibility of an early diagnosis could also improve the efficiency of treatment of maternal hyperglycemia to prevent macrosomia (80). Yet, NIPT is not routinely available for *GCK*-MODY but these preliminary results are promising, particularly those based on relative haplotype dosage estimation. This latter approach will benefit from the development of novel high-throughput sequencing technologies based on long read sequencing, facilitating the reconstruction of haplotypes.

Is Insulin Therapy Effective in Women With *GCK*-MODY?

Although it is currently recommended to institute insulin therapy in *GCK*-MODY women whose babies are at high risk of macrosomia, this strategy has not been implemented in routine practice (41). Thus, in the majority of reported cases, insulin therapy was instituted on the basis of maternal capillary blood glucose upon diet, as recommended in "common" gestational diabetes, or on the discovery of macrosomia by routine US (41) (**Figure 1**). Moreover, no prospective study as defined the best modalities of the treatment and, outside pregnancy, no effect of insulin therapy on HbA_{1c} levels was observed (37–39).

In pregnant women with *GCK*-MODY, scarce reports suggested that insulin therapy may be effective. During two consecutive pregnancies, one woman was treated with insulin, 1 U/kg/d from 10-12 gestation weeks, with normalization of fructosamine values. She delivered a small-for-gestational age (1^{st} percentile) unaffected baby, and a normal weight (30^{th} percentile) affected baby (68). In another report, one woman treated with 1.43 U/kg/d at 30-38 gestation weeks delivered a normal weight (25^{th} percentile) unaffected baby (83). Also, a Japanese study reported that the mean birth weight was lower in unaffected babies born to insulin-treated vs. diet treated mothers [(52), **Table 1**].

However, several retrospective studies have assessed the respective effects of fetal genotype and of treatment with diet or insulin on pregnancy outcomes, and showed that the main determinant of offspring birth weight and of the risk of macrosomia was the fetal genotype, not the treatment of the mother (**Table 1**). Specifically, birth weights were higher in non-affected vs. affected babies, irrespective of the treatment (insulin vs. diet) (41, 42, 50–52). In all studies but one, treatment with insulin did not significantly lower birth weight of affected or unaffected offspring (**Table 1**). In one study, LGA (51 vs. 26%) and neonatal hypoglycemia (24 vs. 3%) were even more frequent in offspring of insulin-treated vs. diet-treated mothers (41). Insulin therapy was also associated with undesirable side effects. The occurrence of maternal hypoglycemia in 56% of the women, including severe hypoglycemia in 23%, was reported in one series (42). Moreover, in almost all studies, insulin therapy was associated with lower gestational age at birth, and with a higher incidence of labor induction and Cesarean deliveries, likely reflecting obstetricians' concerns when pregnant women are treated with insulin (50).

Several hypotheses have been made to explain the poor results of insulin therapy in this context. All these studies were retrospective and some spanned over several decades. Selection bias likely occurred, leading to treat more frequently women with a more pronounced hyperglycemia and/or with already large babies, as suggested by the higher rate of macrosomia in offspring born to insulin-treated mothers in one study (41). Also, insulin dosage was highly variable, ranging 0.1-1.5 U/kg/d, and often may have been insufficient to lower blood glucose levels to the strict targets required to prevent accelerated fetal growth (85). Indeed, one series reported fasting and post-meal blood glucose profiles, recorded in 16 insulin-treated mothers with *GCK*-MODY during the first and the third trimester of pregnancy, that demonstrated glucose values well above recommended targets in almost all women (41). Gestational age at initiation of insulin therapy was also highly variable (from pre-conceptual to 38 weeks of gestation), while it has been suggested that early normalization of maternal blood glucose is necessary to prevent macrosomia reviewed in (80). Lastly, one further barrier could be the up-regulated counterregulation in *GCK*-MODY that will prevent strict normalization of blood glucose values. It has been suggested that high insulin doses, e.g. ≥ 1 U/kg/d., may be needed to normalize blood glucose, but this may be at the cost of a high risk of hypoglycemia. These difficulties are well recognized and account for the recommendation to induce delivery at 38 weeks of gestation in *GCK*-MODY women treated with insulin (18, 50).

IN SEVERAL AREAS THERE COULD BE OPPORTUNITIES TO IMPROVE THE MANAGEMENT AND THE PROGNOSIS OF PREGNANCY IN WOMEN WITH *GCK*-MODY

Improving the Diagnosis of *GCK*-MODY in the Women Before and During Pregnancy

Since patients are asymptomatic the diagnosis of *GCK*-MODY relies on systematic screening. Information about monogenic diabetes should be delivered to healthcare workers and in the general population. The fortuitous discovery of a mild hyperglycemia should not be neglected. First-degree relatives of probands with *GCK*-MODY should systematically be screened.

During pregnancy, early diagnosis of *GCK*-MODY is difficult since currently only women with risk factors for gestational diabetes are screened in the first trimester. Also, the performance of algorithms to select hyperglycemic women to be genetically screened should be evaluated prospectively in multiethnic studies.

Improving the Diagnosis in the Fetus

In pregnant women with a confirmed *GCK*-MODY, non-invasive fetal genotyping will hopefully replace the US-guided management when routinely available. This will considerably

lighten the care and the follow-up of pregnancy in women whose fetuses have inherited the mutation (**Figure 1**).

The Best Modalities and the Efficiency of Insulin Therapy Remain to be Determined

As fetal growth is highly sensitive to mild degrees of hyperglycemia, treatment of maternal hyperglycemia, at the earliest during pregnancy and with strict glycemic targets, is mandatory to decrease adverse events (86). However, decreasing blood glucose values with insulin is associated with a high risk of maternal hypoglycemia, including severe episodes (87). Continuous glucose monitoring during pregnancy improves maternal glycemia and pregnancy outcomes, and is now recommended in pregnant women with type 1 diabetes (88). Moreover, it has been suggested that closed-loop and sensor-augmented pump insulin delivery could be efficient to lower blood glucose levels without increasing the risk of severe hypoglycemia in pregnant women (89). Whether this could be used to overcome the up-regulated counterregulation without increasing the risk of hypoglycemia in women with *GCK*-MODY is not known. Since it is difficult to perform studies in pregnant women, one may suggest to first test the feasibility of this approach in patients with *GCK*-MODY outside pregnancy.

Yet, it is unlikely that other treatments could be used. Sulfonylureas are not a good choice, since they cross the placental barrier, stimulate fetal insulin secretion and are responsible for macrosomia and neonatal hypoglycemia (90). Theoretically, metformin, alone or in association with insulin, could be used. However, metformin crosses the placental barrier, and concerns have been raised about undesirable long-term effects in exposed children (91). Moreover, its potential benefit has not been assessed in patients with *GCK*-MODY outside pregnancy.

CONCLUSION

GCK-MODY is a quasi-experimental human model that allowed to define the respective roles of maternal hyperglycemia and fetal genotype on fetal growth, and to confirm the central role of fetal insulin secretion in fetal growth. It is also a unique situation suggesting that fetal exposure to maternal hyperglycemia is not always responsible *per se* for late adverse consequences in the offspring. Non-invasive fetal genotyping is a major advance in the care of *GCK*-MODY women, since it will allow determining those women whose diabetes should be treated during pregnancy. Challenges persist in the accurate identification of women with *GCK*-MODY before or in early pregnancy, and in the definition of therapeutic modalities during pregnancy. Multicenter studies or registers could be useful to improve our knowledge in these fields.

AUTHOR CONTRIBUTIONS

JT, CS-M, and CB-C wrote the draft of the manuscript. All authors contributed to the writing and the reviewing of the manuscript. All authors approved the final version of the manuscript.

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Not Enough Fat: Mouse Models of Inherited Lipodystrophy

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Lipodystrophies belong to the heterogenous group of syndromes in which the primary defect is a generalized or partial absence of adipose tissue, which may be congenital or acquired in origin. Lipodystrophy should be considered in patients manifesting the combination of insulin resistance (with or without overt diabetes), dyslipidemia and fatty liver. Lipodystrophies are classified according to the etiology of the disease (genetic or acquired) and to the anatomical distribution of adipose tissue (generalized or partial). The mechanism of adipose tissue loss is specific to each syndrome, depending on the biological function of the mutated gene. Mice models, together with cellular studies have permitted clarification of the mechanisms by which human mutations deeply compromise adipocyte homeostasis. In addition, rodent models have proven to be crucial in deciphering the cardiometabolic consequences of the lack of adipose tissue such as NAFLD, muscle insulin resistance and cardiomyopathy. More precisely, tissue-specific transgenic and knockout mice have brought new tools to distinguish phenotypic traits that are the consequences of lipodystrophy from those that are cell-autonomous. In this review, we discuss the mice models of lipodystrophy including those of inherited human syndromes of generalized and partial lipodystrophy. We present how these models have demonstrated the central role of white adipose tissue in energetic homeostasis in general, including insulin sensitivity and lipid handling in particular. We underscore the differences reported with the human phenotype and discuss the limit of rodent models in recapitulating adipose tissue primary default. Finally, we present how these mice models have highlighted the function of the causative-genes and brought new insights into the pathophysiology of the cardiometabolic complications associated with lipodystrophy.

Keywords: adipocyte, lipodystrophy, insulin resistance, cardiometabolic abnormalities, diabetes

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INTRODUCTION

Inherited lipodystrophies belong to the heterogeneous group of syndromes characterized by a lack of adipose tissue (AT) associated with insulin resistance, hypertriglyceridemia, and non-alcoholic fatty liver disease (NAFLD) (1, 2). According to the severity and the anatomical distribution of AT, lipodystrophy could be generalized or partial (3).

Generalized lipodystrophy or Berardinelli–Seip congenital lipodystrophy (BSCL) is characterized by an almost complete lack of AT from birth or early infancy. Severe insulin

resistance (assessed by the presence of acanthosis nigricans) progresses to overt diabetes during the teenage years or later. BSCL is a rare heterogeneous recessively inherited disorder (4).

Partial lipodystrophies are characterized by a stereotypic pattern of AT loss affecting the limbs and normal or excess fat on the face and the neck (3). The reason for the fat depot phenotypical differences remains unknown. Metabolic features range from asymptomatic impaired glucose tolerance with mild dyslipidemia to severe insulin resistance, diabetes and NAFLD (5). The familial partial lipodystrophy (FPLD) syndromes are usually transmitted according to an autosomal dominant mode of inheritance (3).

The mechanism of AT loss is specific to each lipodystrophic disorder, depending on the biological function of the mutated gene (6) (**Figure 1**). Lipodystrophies are rare conditions and clinical studies are difficult to conduct. In BSCL, the absence of AT may occur at birth or in infancy and is the first sign of the condition. Therefore, historically, it has been difficult to know whether the lack of AT was related to a developmental defect or to a rapid and massive loss of mature adipocytes. Mice models, together with cellular studies allowed the identification of the mechanisms by which human mutations can deeply compromise AT homeostasis. Similarly, rodent models appeared to be crucial to decipher the cardiometabolic consequences of the lack of AT such as NAFLD, muscle insulin resistance and cardiomyopathy. More precisely, tissue-specific genetically modified mice brought new tools to distinguish phenotypical traits that are the consequences of lipodystrophy or that can be attributed to cell autonomous mechanisms.

In this review, we discuss how mouse models of inherited lipodystrophy have demonstrated the central role of white AT (WAT) in energetic homeostasis in general, including insulin sensitivity and lipid handling in particular. Then, we present how the different mice carrying gene deletions involved in generalized and partial lipodystrophies highlight the function of these genes and brought new insights into the pathophysiology of the cardiometabolic complications associated with these pathologies.

LIFE WITH NO FAT: MICE MODELS OF GENERALIZED LIPODYSTROPHY

No Fat, No Good

In 1993, Spiegelman's group have tested the effect of limiting AT expansion on metabolic health in the context of obesity (7). To this end, they have generated mice expressing low levels of a diphtheria toxin under the adipocyte-specific aP2 promoter. Whereas adipose-specific expression of the diphtheria toxin has no effect in lean mice, it resulted in a strong reduction of adiposity and resistance to weight gain in obese mice. Importantly, the mice displayed severe hypertriglyceridemia and liver steatosis demonstrating that impairing AT expansion has a deleterious effect on metabolic health.

A few years later, three mouse models of generalized lipodystrophy have been generated by modifying the expression of key transcription factors involved in adipogenesis: the adipocyte-specific over-expression of the nuclear mature form of SREBP1c (aP2-nSREBP1c) (8), the adipocyte specific expression of a dominant negative protein that binds to C/EBPα (A-ZIP/F mice) (9) and the suppression of C/EBPα expression in the AT (10). All three mice presented with a nearly complete lack of AT, diabetes and hepatic steatosis, displaying therefore all the characteristics of BSCL. Importantly,

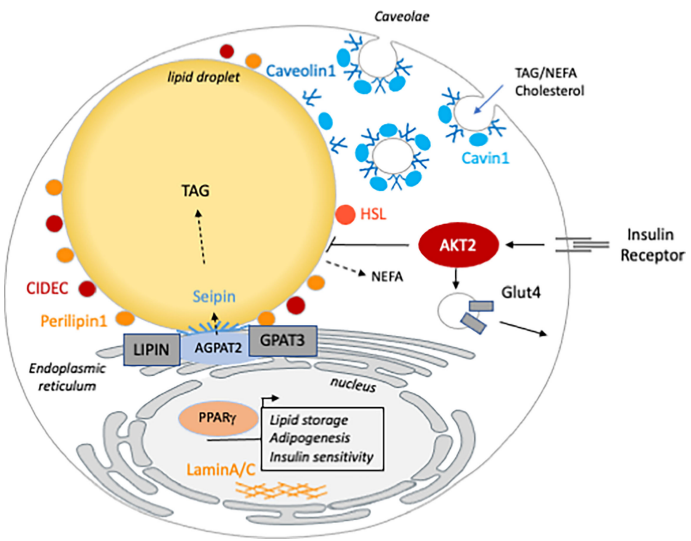

FIGURE 1 | The cellular function of the genes mutated in inherited lipodystrophy. Genes involved in generalized (blue) are involved in TG synthesis (AGPAT2), LD (BSCL2/seipin) or caveolae (Caveolin1 and Cavin1) homeostasis. Partial lipodystrophy causative genes (orange) are involved in different functions of the mature adipocyte. CIDEC, PLIN1 and HSL are LD associated and/or involved in lipolysis regulation. AKT2 and PPARG are both involved in insulin sensitivity. Mutations in LMNA, the gene encoding the nucleophilic lamins A/C, are the most frequent cause of FPLD but how they lead to lipodystrophy remains poorly understood.

surgical transplantation of AT in A-ZIP/F mice (11), as well as leptin supplementation of aP2-nSREBP1c mice (12), strongly improved the insulin-resistance and liver steatosis, pointing out that the lack of AT is central to the development of the metabolic complications associated with lipodystrophy. Those mice could be considered as the first models of generalized lipodystrophy even though the genetic cause was not the one identified in humans. Later on, the identification of the genes responsible for BSCL in humans led to the development of new rodent models of generalized lipodystrophy.

The Triglyceride Synthesis Key Player AGPAT2 Controls Adipocyte Maintenance

1-acyl-sn-glycerol-3-phosphate acyltransferases (AGPAT) are key enzymes involved in lipid synthesis. The isoform AGPAT2, highly expressed in the AT, catalyses the acylation of lyso-phospatidic acid (LPA) to produce phosphatidic acid (PA) that will subsequently enter the triglyceride (TG) or phospholipid synthesis. In humans, biallelic AGPAT2 mutations cause BSCL1 (13). Total AGPAT2 KO mice present with virtually no white nor brown AT (14). The AGPAT2 deficient mice are hyperglycemic, hyperinsulinemic, hypoleptinemic, insulin-resistant and display liver steatosis. Indirect calorimetry studies revealed that these mice have a constant respiratory quotient along the day suggesting that they are metabolically inflexible and unable to switch from one substrate to another according to the nutritional status (14). Interestingly, AGPAT2 re-expression in the liver of total KO mice did not rescue the massive liver steatosis (15) whereas leptin replacement did, as well as it improved glucose homeostasis (16). This reinforces the central role of leptin deficiency in the pathophysiology of BSCL. This strongly suggests that lipodystrophy was the cause of the metabolic disorders described in AGPAT2 KO mice. Therefore, several studies intended to describe AGPAT2 function in AT.

Cellular studies have supported that AGPAT2 is necessary for adipocyte differentiation, suggesting that the absence of WAT in AGPAT2 KO was the result of an altered adipogenesis (17, 18). Surprisingly, the characterization of the AT in foetuses, at birth and in the first week of life, revealed that the AGPAT2 KO newborns have normal AT that is rapidly lost. Another study confirmed that WAT depots are normal until AGPAT2 KO mice were 1-week-old but completely absent in aged mice, supporting that AGPAT2 is required for mature adipocytes maintenance (19). Indeed, rather than a developmental issue, AGPAT2 deficiency induces autophagy, inflammation and massive cell death (20). Importantly, the apoptosis induction was associated with a strong accumulation of PA, an increase in phosphatidylcholine (PC) and a decrease in phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylglycerol (PG). As PC increases and PE remains unchanged, PE/PC (20) ratio likely drops thereby inducing cellular stress and cell death as previously demonstrated (21). Of note, AGPAT2 KO adipocytes are devoid of caveolae (20), a subclass of membrane microdomains involved in adipocyte fat storage and whose deficiency is associated with lipodystrophy (see later).

Interestingly, Lipin-1, the enzyme that catalyses the transformation of PA into diacylglycerol (DAG) has been involved in lipodystrophy in mice only (22), not in human (23). Spontaneous loss-of-function mutations in the gene encoding Lipin-1 have been identified in the fatty liver dystrophic (FLD) mice, characterized by a massive liver steatosis in the pre-suckling period (24) and a severe lipodystrophy associated with glucose intolerance in adult animals (25). In mice, adipocyte restricted deletion of Lipin-1 strongly affects adipocyte TG synthesis, leads to PA accumulation, and induces lipodystrophy (26). Lipin-1 is both a co-regulator DNA binding factor and a PA-phosphatase enzyme involved in TG synthesis. Importantly, a unique mouse model has been generated with a truncated Lipin-1 lacking the lipid synthesis activity but retaining the DNA binding domain (27). Those mice display severe adipose tissue loss supporting that the TG synthesis activity of Lipin-1 is crucial for adipocyte maintenance. Taken together with the lessons from AGPAT2 deficiency, these findings demonstrate that the TG synthetic capacity of the adipocyte is a crucial determinant of adipocyte maintenance.

BSCL2 Encodes the Mysterious Protein Seipin

Mutations in the gene *BSCL2* have been the first genetic explanation for generalized lipodystrophy in humans (28). *BSCL2* encodes seipin whose biological function remains poorly understood, especially in adipocytes. *In vitro* studies report that seipin is involved in lipid droplet (LD) homeostasis and in LD/ER (endoplasmic reticulum) contact sites [for review (29)]. *BSCL2* transcripts are highly expressed in brain, AT and testis. Different animal models have been generated to better describe the pathophysiology of BSCL2. Initially, three total and constitutive seipin deficient (SKO) mice have been studied and showed similar characteristics (30–32). 8 to 12-week-old mice display a near-complete lack of WAT (90% reduction), insulin-resistance and hepatic steatosis. A loss of 60 to 50% of brown AT (BAT) mass is also observed. Unexpectedly, SKO mice are hypotriglyceridemic, in contrast to human BSCL2 patients who display elevated TG levels. One study proposed that this low TG levels might be due to an increase in TG-rich lipoprotein uptake in the liver of SKO mice (30). Of note, BSCL2 deficient rat are hypertriglyceridemic suggesting that rat could be a better model to study lipoproteins in the context of lipodystrophy (33). In the absence of energy storage, these mice are intolerant to fasting and exhibit metabolic inflexibility (31, 34). SKO mice show a decrease in TG and an increase in glycogen in skeletal muscles (35). Seipin deficiency induces renal dysfunction associated with elevated glycation and TG levels in SKO glomerular area (36). Since AT transplantation and leptin replacement improve the renal function, the kidney phenotype is likely a consequence of the lipodystrophy and not a cell autonomous function of seipin. A recent study reported a pancreatic phenotype characterized by a beta-cell hypertrophy and an alteration of the insulin secretion profile in response to a glucose bolus (37). Intriguingly, this study showed that the heterozygous deletion of seipin is sufficient to lead to beta-cell dysfunction whereas it does not alter the AT

mass, suggesting a cell autonomous action of seipin in beta-cells. Further studies with a pancreatic-specific deletion of seipin are needed to confirm this hypothesis.

Three studies have also reported the rapid development of diabetic cardiomyopathy characterized by left ventricular hypertrophy, cardiac insulin resistance and diastolic dysfunction (38, 39). We have shown that in SKO mice, cardiac dysfunction is associated with hyperglycemia, cardiac glucose overload and more precisely with a chronic activation of the hexosamine biosynthetic pathway (HBP). Interestingly, SGLT2 inhibitor (dapagliflozin) treatment normalized the plasma glucose level, decreased the chronic activation of the HBP, and improved the cardiac phenotype of SKO mice (38). The second study proposed that cardiac dysfunction is related to chronic activation of FA oxidation in SKO heart as a consequence of uncontrolled lipolysis. Indeed, inhibition of adipose tissue TG lipase (ATGL) ameliorates the lipodystrophic phenotype and consequently corrects cardiac dysfunction (39). The last report incriminates changes in the phosphorylation levels of the sarcomeric protein Titin (40). In this report, cardiac specific deletion of seipin did not lead to heart abnormalities suggesting that cardiomyopathy is a consequence of lipodystrophy and not an autonomous cardiac dysfunction (40).

In order to address the question of the central role of the adipocyte seipin deficiency and subsequent lipodystrophy in the pathophysiology of BSCL2, several genetic animal models have been created. First of all, BSCL2 re-expression specifically in the adipocytes, through the aP2 promoter, is sufficient to correct the SKO mice lipodystrophy, insulin resistance and liver steatosis (41). At the opposite, liver-specific seipin deficiency (42, 43) does not induce liver steatosis nor insulin resistance, discarding an autonomous role of seipin in the hepatocyte at the origin of the liver complications reported in BSCL2 patients. Adipocyte-specific seipin deletion, either under the aP2 promoter (44) or the AdipoQ promoter (45), leads to progressive lipodystrophy. Under the aP2 promoter, the lipodystrophy is associated with all the associated metabolic complications (insulin resistance, glucose intolerance and liver steatosis) (44). In the second model, the metabolic complications are severely marked only under high-fat diet (HFD). Regarding the origin of lipodystrophy in the BSCL2 phenotype, *in vitro* experiments support that seipin is crucial for normal adipogenesis (46). However, adipogenesis impairment cannot fully explain the SKO severe lipodystrophy, as in these mice, we reported a loss of WAT mass and a decrease in circulating adiponectin levels between 4 to 12 weeks of age (47). Consistently, inducible adipose-specific seipin deletion compromises adipocyte survival and results in elevated basal lipolysis, leading to progressive AT loss (48). Therefore, seipin might play a role in both, adipocyte differentiation and maintenance of the full mature adipocyte phenotype.

The Unexpected Involvement of Caveolae

In humans, mutations in *CAV1* encoding the caveolae protein caveolin-1, lead to a near complete loss of subcutaneous and visceral AT, associated with insulin resistance and dyslipidemia, therefore referred as BSCL3 (49). BSCL4 is caused by loss-of-function mutations in *CAVIN1/PTRF* (Polymerase I and Transcript Release Factor) encoding a required protein for caveolae biogenesis which regulates the expression of caveolins (50–52).

Caveolin-1 is a key structural protein of caveolae, omega-shaped membranous invaginations, that, together with cavin adaptor proteins, decorated almost 30% of adipocyte plasma membrane (53). Although caveolin-1 and/or cavin-1 deficiency leads to complete loss of caveolae structures, mice are still fertile and viable (54–56). Despite normal AT depots at birth, caveolin-1 null mice display a progressive lipoatrophy aggravating with age, although developing with a slightly different time frame depending on the KO model considered, characterized by the loss of hypodermal fat layer and generalized reduction of all WAT depots, hypertriglyceridemia but very mild insulin resistance as soon as 3 months of age (54, 57). CAV1 KO mice are moreover resisting to the development of obesity when fed a HFD (54, 58). They also exhibit elevated triglycerides and reduced leptin and adiponectin and overt diabetes only developed in the context of prolonged HFD (59). Whereas the leanness of CAV1 KO mice has been shown to be independent of altered energy expenditure, food intake or intestinal absorption (54), their complex metabolic phenotype has been related to metabolic inflexibility and mitochondrial dysfunction (60).

The generation of an adipocyte-specific KO of caveolin-1 was unsuccessful since the efficient exosomal trafficking of caveolin-1 from neighbouring endothelial cells compensates the adipocyte caveolin-1 deficiency (61).

Cavin-1 invalidation reproduced typical BSCL phenotype with significant fat loss, histological abnormalities of AT including marked fibrosis, and a significant decrease in circulating levels of adiponectin and leptin. From a metabolic point of view, the mice also show glucose intolerance, hepatic and muscular insulin resistance and hypertriglyceridemia (55, 62). The similarities of the lipodystrophic phenotypes displayed by CAV1 and CAVIN1 KO mice tend to incriminate the absence of caveolae structures in the development of metabolic alterations. Nonetheless, we can exclude that those cellular mechanisms regulated by nuclear and/or cytosolic cavin-1 also participate to this metabolic phenotype, a speculation that would require further investigations. Overall, distinguishing between the specific role attributed to individual caveolae-forming proteins and the ones linked to caveolae microdomains is still technically challenging, given the fact that they are intrinsically linked, emphasizing the need to explore alternative molecular models for a better understanding of their respective contribution (63).

Among the metabolic pathways impacted by caveolae disappearance, the localization and internalization of the insulin receptor within these membrane microdomains (59) early identified caveolin-1 as a positive regulator of the insulin signalling pathway. Moreover, as a lipid-binding protein (64), caveolin-1 is thought to participate to lipid trafficking, between plasma membrane and the LD (65, 66) and to modulate LD phospholipid and protein surface composition (67). Caveolin 1

deficiency alters fatty acid uptake (68, 69) but adipocyte cell surface caveolae might also be sites of local triglycerides synthesis (70). In adipocytes, we further demonstrated a reciprocal regulation of membranous caveolae density and fat cell LD storage, highlighting caveolae as mediators of lipid-driven fat cell size adaptation and expandability (71).

Despite no major abnormalities in energy balance reported in CAV1 KO mice, the absence of caveolin-1 has been linked to reduced ability to change substrate use in response to feeding/fasting conditions, which has been referred to metabolic inflexibility (60). Since mature adipocytes are present in young mice and mouse embryonic fibroblasts from CAV1 KO mice differentiate into adipocytes, caveolin-1 is not *per se* required for the formation of new adipocytes (65, 72). Besides, lipoatrophy might result from exaggerated breakdown of WAT stored lipids, since both caveolin-1 and cavin-1 have been both shown to be critical in regulating lipase-induced lipid mobilization (72, 73). Nonetheless, the study of the lipolytic response of isolated adipocytes to beta-3 adrenergic agonists has revealed blunted rather exacerbated lipolysis in CAV1 KO mice (72). Altered response to pro-lipolytic signals in CAV1 null animals results in increased susceptibility to cell death, inflammation and fibrosis in WAT (74). We moreover revealed constitutive adipocyte activated autophagy in the absence of caveolin-1, that associates with altered protein turnover and accelerated protein degradation impairing many metabolic pathways (57). Cellular studies using cultured skin fibroblasts from patients also argue for a role of maladaptative autophagy in the absence of cavin-1 that contributes to insulin resistance (75). It remains so far unclear whether such degradative process directly impacts adipocyte cell death and/or renewal as a primary defect of lipodystrophy or whether it develops as an adaptive mechanism to counteract adipocyte dysfunction.

In summary, caveolin-1 deficient adipocytes have to face with a metabolic situation characterized by defective fatty acid mobilization and an altered insulin-dependent nutrient supply which both likely converge to induce autophagy. These metabolic stresses could have an additional impact on WAT remodelling processes and the development of an inflammatory state, that, altogether, may favour and contribute to the development of lipodystrophy.

Generalised Lipodystrophy Is the Cause of Metabolic Complications

Collectively, BSCL mice models display severe metabolic complications. For BSCL2, adipocyte specific models and inducible deletion recapitulate most of the features of the total SKO mice. In addition, for AGPAT2 and BSCL2 KO mice, leptin replacement or AT surgical implantation strongly improved the metabolic phenotype including insulin resistance, liver steatosis and renal injuries. Recently, Kahn's lab generated adipocyte-specific tamoxifen inducible insulin receptor deletion (Adipo-ind-IRKO) and demonstrated that 3 days after tamoxifen injection, mice display a massive adipocyte loss and severe insulin resistance (76). Importantly, leptin supplementation prevents the appearance of insulin resistance and liver steatosis, but did not improve adipose tissue mass and quality. Unexpectedly, 30 days after tamoxifen injection, the neo-differentiation of new adipocytes that express the insulin receptor induces a re-increase in AT mass and a correction of glucose homeostasis abnormalities. This report remarkably demonstrated that metabolic health is dynamically determined by AT and that among adipocyte properties, leptin secretion is central in its ability to control metabolic homeostasis.

WHAT DID WE LEARN FROM PARTIAL LIPODYSTROPHY RODENT MODELS?

LMNA, the Most Common Cause of Familial Partial Lipodystrophy

The most frequent genetic cause of FPLD is mutations in *LMNA*, and among them, the R482Q mutation represents 80% of the cases. *LMNA* encodes the nucleophilic A-type lamins, lamin A and lamin C which are generated by different splicing within exon 10 of *LMNA*. Several mice models have been generated to understand how lamin A/C mutations cause FPLD. In LMNA-deficient mice, growth is decreased at two weeks-old and completely stopped at 4-weeks old, and the animals do not survive over 2 months-old. The origin of death is a severe and early muscular dystrophy that leads to posture abnormalities such as scoliosis and major heart dysfunction (77). Those mice also display complete AT loss without metabolic complications (78). Lipodystrophy is suspected to be secondary to the muscular dystrophy but the severity of the phenotype renders this model difficult to interpret. On the other hand, the adipocyte-specific LMNA deficiency leads to a reduction of WAT mass in male and female mice that was associated with mild hyperglycemia and hyperinsulinemia in females (79). Five weeks-HFD feeding leads to a more severe lipodystrophy and a more marked metabolic phenotype characterized by hyperglycemia, hyperinsulinemia, elevated TG and low adiponectin and leptin levels. Importantly, in adipocyte specific LMNA-deficient mice, the AT develops postnatally but progressively disappears from 4 weeks of age, suggesting that LMNA deficiency alters adipocyte maintenance. Consistently, *in vitro* studies support that LMNA deficiency does not impair adipogenesis of mesenchymal stem cells but accelerates lipolysis in differentiated adipocytes. This phenotype is quite consistent with that reported in FPLD patients carrying *LMNA* mutations.

Transgenic mice expressing human *LMNA* with the common R482Q mutation under the adipocyte specific aP2 promoter have been generated (80). Of note, in these mice, the transgene is present in the hemizygous state in addition to the two wild-type copies of the murine *Lmna* gene; while a knock-in introduction of the R482Q mutation would be closer to the situation of FPLD patients. Under HFD feeding, those mice develop lipodystrophy, abnormal AT histology, glucose intolerance, insulin resistance and liver steatosis. Adipose tissue loss consecutive to R482Q mutation introduction is similarly observed in all depots at the exception of the inguinal WAT which is not significantly impacted. The AT distribution and the metabolic phenotype of

the R482Q mice resemble those reported in the adipocyte-specific LMNA-deficient mice. However, the *in vitro* data obtained with stromal cells isolated from the R482Q AT generate different results than those obtained with the mesenchymal cells from adipocyte-specific LMNA-deficient WAT. Indeed, *in vitro*, the R482Q mutation does not modify lipolysis rates but alters adipogenesis. Therefore, whereas the phenotype of mice with the R482Q mutation or adipocyte-specific LMNA deficiency are similar, the mechanism at the origin of lipodystrophy remains elusive.

PPAR Gamma

PPARγ (Peroxisome proliferator-activated receptor gamma) is the master regulator of adipogenesis and total KO of PPARγ is lethal (81, 82). Adipocyte-specific deletion of PPARγ under the AdipoQ gene promoter leads to a nearly complete lack of AT, insulin resistance and massive liver steatosis, *i.e.* generalized lipodystrophy (83). In humans, a dominant-negative mutation in the ligand binding domain of PPARγ (P467L), is associated with severe insulin resistance, diabetes and hypertension (84), and further clinical characterization revealed a FPLD syndrome (85). Surprisingly, mice carrying the equivalent P465L mutation do not develop lipodystrophy nor insulin resistance under chow or HFD (86, 87) but display a change in fat distribution with an increase in subcutaneous fat pads and a decrease in gonadal WAT (86). This unexpected result suggests that in mice, P465L confers leanness rather than pathological lipodystrophy. In order to understand further the pathophysiological effect of the P465L mutation in mice, one group crossed these mice with the obese and hyperphagic leptin-deficient ob/ob mice model (88). The heterozygous P465L mutation on an ob/ob background leads to a reduction of WAT mass, the development of severe insulin resistance, lipid liver accumulation and alteration in post-prandial TG clearance (89). Of note, this was not the case after 16 weeks HFD feeding (89), suggesting that a stronger positive energy balance is needed to reveal the metabolic consequences of limited AT expansion due to P465L dominant-negative mutation of PPARγ. To conclude, whereas it is clear that adipocyte PPARγ deficiency leads to severe lipodystrophy, the FPLD phenotype due to P465L is not easy to recapitulate in mice and it appears that according to the body weight phenotype (lean, mild overweight or obese), this mutation will balance towards healthy leanness or lipoatrophic insulin resistant phenotype.

Perilipin-1

Perilipin-1 is the most abundant LD-associated protein in mature adipocytes and its main biological function is to prevent basal lipolysis and to allow adrenergic stimulated TG hydrolysis (90). Heterozygous loss-of-function mutations in *PLN1* gene have been identified in FPLD patients, causing partial lipodystrophy and severe insulin resistance (91, 92). Functional characterization of three mutations in cellular experiments demonstrated that mutated perilipin-1 fails to repress basal lipolysis and prevents therefore from lipid accumulation (91–93). There are no knock-in mice for any of these mutations but PLN1-deficient mice have been generated. The initial publications characterized the effect of PLN1 deficiency under a mixed C57Bl6J/129 background (94). The WAT collected from $Pln1^{-/-}$ mice displays 70% mass reduction, small and abnormal LD and consistently with perilipin-1 function, elevated basal lipolysis and reduced adrenergic stimulated lipolysis (94). $Pln1^{+/-}$ mice tend to have a reduced WAT mass, which is however not significantly different from their wild-type littermates. When Perilipin-1 deficiency is produced under a pure C57/Bl6 background, the mice similarly display partial lipodystrophy but a more marked insulin resistance along with strong macrophage inflammation in WAT is observed (95). No report examined the phenotype of $Pln1^{+/-}$ under C57/Bl6 background. In summary, in humans, heterozygous frameshift mutations in perilipin-1 cause FPLD, a phenotype recapitulated in mice following homozygous perilipin deficiency.

Hormone-Sensitive Lipase

In humans, bi-allelic null mutations in the *LIPE* gene, encoding the hormone-sensitive lipase (HSL), is associated with a complex AT phenotype including fat redistribution, multiple symmetric lipomatosis (excess fat accumulation) and partial lipodystrophy (96–98). At the metabolic level, the patients display different ranges of metabolic complications such as dyslipidemia, hepatic steatosis and systemic insulin resistance. In most cases, the consequence of HSL deficiency induces a late-onset disease with an age of diagnosis ranging from 23 to 76 years old. This complex phenotype could be attributed to the central role played by HSL in fat mobilization or lipolysis. Numerous studies on HSL-deficient mice are useful to further understand the pathophysiology involved.

Total HSL-deficiency strongly alters WAT properties resulting in blunted catecholamine stimulated lipolysis (99), heterogenous size of adipocytes (ranging from hypertrophic to abnormally small fat cells), DAG accumulation (100) and low adiponectin expression (101). The massive DAG accumulation likely contributes to the AT phenotype although this has not been formally demonstrated. In addition, AT stems cells isolated from a lipodystrophic patient carrying a bi-allelic *LIPE* null variant display impaired adipogenesis *in vitro* (97). However, it remains unclear how the clinical features can range from lipomatosis to partial lipodystrophy. Transgenic expression of human HSL gene restored normal adipose tissue mass, histology and circulating leptin levels (102). The glucose homeostasis status generated quite a lot of discussion and whereas some reports suggested that HSL deficiency might increase insulin sensitivity (101), others supported an impairment in glycemic control (103, 104), including a default in insulin secretion (105). Interestingly, whereas one study has shown that under short a HFD exposure (3 weeks), HSL deficiency protects the animals from the adverse effect of HFD (106); in an ob/ob background, HSL deficiency worsens the glucose homeostasis dysfunction (107). A recent study helped us to reconciliate these different findings. At 3 months of age, the HSL-KO mice display normal body weight, normal AT, and improved insulin sensitivity as compared to control mice (108). By contrast, 8-months old animals display lower body weight, progressive lipodystrophy along with AT macrophage infiltration, liver steatosis and insulin

resistance (108). Finally, using liver and adipocyte-specific deletion of HSL, they demonstrated that only adipocyte HSL-deficiency recapitulates the whole phenotype reported in the global KO mice (108). Taken together, these studies collectively reported that, according to the age and energy balance status, HSL-deficiency might be temporary protective, but appears deleterious with ageing and increased energetic supply. This is compatible with the late-onset of the disease in humans.

CIDEC

A female patient presenting with partial lipodystrophy insulin resistance and diabetes was found to be homozygous for a mutation (E186X) that leads to a premature truncation in the LD protein, cell death-inducing DFFA-like effector C (CIDEC aka FSP27) (109). Histological analysis of her AT revealed the presence of many multilocular adipocytes within the subcutaneous WAT depot. In cell experiments, the truncated protein does not surround the LD and fails to increase LD size, at the opposite of the WT protein (109). However, this mutation is not a loss-of-function and the protein still carry the CIDE-N domain. No mouse model of E186X-CIDEC has been generated, but CIDEC-KO mice are available. Interestingly, the white adipocytes of CIDEC-KO mice display multilocular LD phenotype, with increased mitochondria number and elevated FA oxydation (110). This adipocyte morphology resembles the one described in the lipodystrophic patient. However, under chow diet, CIDEC deficiency limits gain weight from 16 weeks and decreases random fed glycemia as well as it improves glucose tolerance. Under HFD, CIDEC-deficiency prevents weight gain and the appearance of the metabolic complications associated with obesity such as glucose intolerance and insulin resistance (110). The similar protecting effect was reported when CIDEC-deficient mice were crossed with ob/ob mice: the animals were leaner and have improved glucose homoeostasis (111). Similarly, to CIDEC KO mice, adipocyte-specific CIDEC deletion using aP2-CRE mice leads to a similar multilocular adipocyte phenotype. HFD-feeding of adipocyte-specific CIDEC deletion prevents from body weight gain but leads to insulin resistance, elevated plasma TG and FFA, and liver steatosis (112). CIDEC expression is 10-fold increase in the liver of these animals and liver-specific CIDEC overexpression has been shown to induce liver steatosis (113). In order to reconciliate these data, further studies on adipocyte versus liver CIDEC contribution are needed. Of note, the adipocyte specificity and the efficiency of aP2 CRE has been challenged (114) and might explain in part this unexpected phenotype.

AKT2

One single family has been identified with partial lipodystrophy and severe insulin resistance that is due to a missense mutation in the *AKT2* gene encoding the key insulin signalling ser/thr kinase. This R274H substitution shows an autosomal dominant transmission and exerts a dominant negative-effect on wild-type AKT2, compromising insulin signalling in hepatocytes and adipocyte cell lines (115). AKT2-deficient mice display severe systemic and muscle insulin resistance (116, 117) and progressive lipodystrophy affecting all WAT depots (117). Interestingly, adipocyte-specific deletion of AKT2 is sufficient to recapitulate severe lipodystrophy, liver steatosis and hyperglycemia and hyperinsulinemia despite normal glucose tolerance (118). The severe AT loss in this model is consistent with the phenotype reported in Adipo-ind-IRKO (76) (cf 2.5) supporting a central role of adipocyte insulin signalling in controlling adipocyte good health. On the other hand, the fact that adipocyte-specific deletion of AKT2 does not impair glucose tolerance whereas insulin receptor deletion does, and given that lipodystrophy is similarly severe in both mice, is quite unexpected. Both models display increased beta-cell mass, but as the insulin receptor deletion is inducible and leads to rapid AT loss and lipid spill-over, it is possible to speculate that an adaptative response in constitutive AKT2-deficient mice induces a stronger hyperinsulinemia that compensates for insulin resistance and allows normal GTT. However, in refed conditions, AT deletion of AKT2 leads to hyperglycemia. In conclusion, both models demonstrate that adipocyte insulin signalling control AT properties and systemic glucose homeostasis. Regarding AKT2, whereas there are so far no mouse model harbouring the R274H mutation described to lead to human partial lipodystrophy and severe insulin resistance, adipocyte-specific AKT2 deficiency recapitulates lipodystrophy associated with impaired glucose homoeostasis.

IT IS NOT THAT EASY TO RECAPITULATE FPLD

Most of the mice models of FPLD, generated either by loss-of-function mutations or knock-in of FPLD genes, display healthy leanness, under chow diet. At the opposite, under extremely obese background as for ob/ob mice, limited AT expansion leads to severe metabolic complications. Under HFD conditions, the results are more variable, since short HFD-fed mice display healthy resistance to weight gain whereas longer HFD exposure unmasks insulin resistance, glucose intolerance and liver steatosis. To this extent, the recent longitudinal study reporting a time course in HSL KO mice is very informative and supports the idea that the metabolic consequences are dependent on the storage imbalance. Thus, mice can cope with lower adipose storage capacity when facing moderate energy excess whereas, in conditions of severe hyperphagia (ob/ob) or chronic HFD, the limit of expansion of AT is reached leading to subsequent metabolic complications. Moreover, the analyses of rodent models for FPLD tend to suggest that mice can cope more easily with limited expansion of AT than humans. The example of P465L-PPARγ mice is striking: those mice develop insulin metabolic complications only under very high energy intake (ob/ob background), whereas P467L patients are diabetic. One important element that should be considered here is the heterogeneity of the different fat pads behaviour. Indeed, in humans, FPLD is characterized by loss of limbs WAT but the mass of the other fat pads, including truncal WAT is either unchanged or increased. The reason for this difference in fat pad behaviour remains unknown. In most of the rodent models discussed here, the AT loss is similar between the different

WAT depots, which is already a strong difference with humans. Furthermore, in R482Q-LMNA and P465L-PARγ, the inguinal fat pad is less severely affected. This might explain the difference in the severity of the phenotypes. It is now well established that rodent and human fat pads display strong differences. The rodent gonadal WAT which has been used in most of the studies presented here as visceral AT, is very small in human where visceral fat is more represented by mesenteric and omental WAT (119). Mice inguinal WAT is largely used to study subcutaneous WAT but human subcutaneous fat pads are divided into upper and lower subcutaneous WAT questioning the relevance of using inguinal WAT as a mirror depot of human lower subcutaneous WAT (120). All these key questions call for caution in interpretating rodent experiments with respect to WAT heterogeneity. The recent single cell/nucleus studies in human and mice should bring new valuable information regarding the diversity of progenitors and mature adipocytes in the different fat pads (121, 122).

CONCLUSION AND PERSPECTIVE

Collectively, generalized lipodystrophy mouse models strongly demonstrate that the lack of AT storage leads to ectopic fat deposition, triggering the development of diabetes mellitus. Importantly, NAFLD, diabetic cardiomyopathy, kidney disease and beta-cell dysfunction have been largely studied in BSCL rodent models and further demonstrate the implication of AT failure in the development of cardiometabolic diseases. In addition, the use of adipose-specific tools strongly demonstrates that, most of the time, the metabolic abnormalities are linked to the specific deficiency of the BSCL gene in the adipocytes and that adipocyte implementation is sufficient to restore normal insulin sensitivity, glucose tolerance and to correct liver steatosis. It is therefore obvious that complete or major lack of AT is deleterious for metabolic health and that rodent models are very useful. However, as discussed earlier, it is more difficult to predict the consequences of limited adipose expansion capacity. Indeed, in FPDL models the frontier between leanness and lipoatrophic diabetes is not always obvious and seems to depend on the level of positive energy balance. Based on the data reported here, we would propose that ob/ob background is probably the option to unmask adipose failure phenotype for FPLD genes. The phenotype of FPLD mice also raises the question of what is the rodent fat pad that represents the best omental AT, i.e., typical visceral fat in human, and similarly for gluteofemoral, i.e., subcutaneous WAT in human. In conclusion, rodent models are useful but not perfect tools and *in vitro* studies are crucial to decipher the individual function of the genes involved in inherited lipodystrophies.

AUTHORS CONTRIBUTIONS

SL, JM, and XP wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Generation and Characterization of iPS Cells Derived from APECED Patients for Gene Correction

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APECED (Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal-Dystrophy) is a severe and incurable multiorgan autoimmune disease caused by mutations in the *AIRE* (autoimmune regulator) gene. Without functional AIRE, the development of central and peripheral immune tolerance is severely impaired allowing the accumulation of autoreactive immune cells in the periphery. This leads to multiple endocrine and non-endocrine autoimmune disorders and mucocutaneous candidiasis in APECED patients. Recent studies have suggested that AIRE also has novel functions in stem cells and contributes to the regulatory network of pluripotency. In preparation of therapeutic gene correction, we generated and assessed patient blood cell-derived iPSCs, potentially suitable for cell therapy in APECED. Here, we describe APECED-patient derived iPSCs's properties, expression of AIRE as well as classical stem cell markers by qPCR and immunocytochemistry. We further generated self-aggregated EBs of the iPSCs. We show that APECED patient-derived iPSCs and EBs do not have any major proliferative or apoptotic defects and that they express all the classical pluripotency markers similarly to healthy person iPSCs. The results suggest that the common AIRE R257X truncation mutation does not affect stem cell properties and that APECED iPSCs can be propagated *in vitro* and used for subsequent gene-correction. This first study on APECED patient-derived iPSCs validates their pluripotency and confirms their ability for differentiation and potential therapeutic use.

Keywords: iPSC, pluripotency, autoimmunity, APECED, cell therapy

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INTRODUCTION

Autoimmune-Polyendocrinopathy-Candidiasis-Ectodermal-Dystrophy (APECED), also known as Autoimmune Polyendocrine Syndrome type 1 (APS-1), is a rare and severe multiorgan autoimmune disease caused by mutations in Autoimmune Regulator (*AIRE*) (1, 2). APECED (OMIM 240300) is characterized by a triad of manifestations including Addison disease (AD), hypoparathyroidism, and chronic mucocutaneous candidiasis (CMC) (3, 4). Additionally, considerable phenotypic variation of the disease is common including symptoms of varying severity of hypogonadism, hypothyroidism, hypophysitis, type 1 diabetes, vitiligo, and alopecia (5, 6). This syndrome is usually diagnosed during early childhood or adolescence, and it develops progressively to varying manifestations with increasing

severity (7). APECED patient mortality is significantly increased in all age groups due to adrenal, and hypocalcemic crises, and oral squamous cell cancer, autoimmune hepatitis, pneumonitis, and nephritis (6, 8) as well as alcohol-, and accident-related deaths (9). Additionally, a significantly higher mortality is found to correlate with patients manifesting multiple endocrine symptoms (9). At present, there is no cure for APECED, and management consists of life-long hormone replacement therapy, and treatment of chronic candidiasis (6, 8, 10).

APECED is a monogenic disease with the most common AIRE mutation being the so-called "Finn-major" R257X mutation (11). This nonsense mutation results in the carboxyterminal truncation of AIRE leaving it non-functional and altering its subcellular localization (12). AIRE's classical function within the immune system is to act as a transcription-factor like protein in helping release stalled RNA polymerase II and assisting in translation elongation (13). Functional AIRE is essential for the proper development of central and peripheral tolerance (14, 15). Its expression is tightly regulated in tissues of the immune system such as the thymus and lymph nodes as well in peripheral lymphoid tissues such as within the bone marrow (16, 17). The establishment of immune self-tolerance takes place in the thymus, where AIRE orchestrates the promiscuous gene expression of over 3200 tissue-specific self-antigens (TSAs) in medullary thymic epithelial cells (mTECs) (18–20). Defective AIRE leads to impaired expression and decreased surface display of TSAs to naïve T cells by mTECs. This loss of TSA presentation allows the survival of autoreactive T cells and their escape into the periphery from which they attack target tissues and molecules causing autoimmune manifestations (18). Additionally, also B-cell function and tolerance is abrogated as patients already harbor hundreds of neutralizing autoantibodies notably against a wide group of cytokines (21).

Recently, novel findings have shown *AIRE* to be expressed in stem cells, suggesting it has unforeseen functions outside the immune system. Germ cell progenitors have been found to express *AIRE* and its mutations were shown to cause fertility defects both in mice and in APECED patients (22–24). In addition, *AIRE* is highly active in undifferentiated embryonic stem cells (ES cells) and embryos with its expression decreasing during ES cell differentiation (25, 26). In mouse ES cells, knockdown of *Aire* reduces expression of the classical stem cell markers Oct4 and Nanog diminishing ES cell self-renewal potential (26). Moreover, Aire has been shown to activate another pluripotency inducer LIN28 thus suggesting an active contribution in the regulation and maintenance of pluripotency (25).

The development of gene-correction and iPSC technology has opened up opportunities for precision medicine and therapeutic avenues for monogenic diseases such as APECED. Recently, thymic precursor cells generated from human stem cells were shown to form functional thymic epithelial cells capable of supporting normal T cell development *in vivo* (27). AIRE expression has also been detected in human epidermal keratinocytes (28) that bear significantly similar biological attributes to thymic epithelial cells (13). Moreover, in the absence of a thymus, AIRE + keratinocytes have been shown to restore and support the production of mature, functional T cells concurrently promoting the culling of autoreactive cells (29). We propose that lentiviral re-introduction of a functional *AIRE* gene into patient-derived iPSCs, subsequent differentiation into suitable cell type(s) and re-introduction into affected individuals could elicit a curative effect. However, very little is known about the role of stem cells in APECED or their potential in therapeutic approaches and so far no publications have examined patient-derived iPSCs. Thus here, we generated to our knowledge the first APECED-patient derived iPSCs of two patients harboring the *AIRE* R257X mutation. We investigated the effects of the R257X mutation on iPSC proliferation, apoptosis, stem cell marker expression as well as well as the capability to differentiate normally into embryoid bodies (EBs) (30).

MATERIALS AND METHODS

This study was approved by Helsinki and Uusimaa Hospital District (HUS) Ethics Committee of Medicine (HUS/1127/2016) and all participating patients provided written informed consent.

Cell Culture and iPSC Generation

For iPSC induction two adolescent female APECED patients with confirmed homozygous AIRE R257X mutations donated peripheral blood mononuclear cells that were then induced into iPSCs using the CytoTune-iPS 2.1 Sendai Reprogramming Kit (Invitrogen, Thermo Scientific) by Biomedicum Stem Cell Core (BSCC) (Helsinki, Finland). From both donors' inductions approximately 10 iPSC clones were selected and propagated, with one clone per donor used for experiments (termed 137.2. and 138.6). The healthy control cell line HEL24.3 was similarly generated from an age-matched healthy individual at BSCC. After induction, all cell lines were assessed by the BSCC using multiple assays including immunohistochemistry and qPCR (for expression of OCT4, SSEA4, TRA-1-60) and RT-PCR to assess for the removal of transgene vectors (available upon justified request). The results were that all iPSC lines express endogenous pluripotent stem cell surface markers and Sendai virus vectors were absent from all lines thus meeting the common criteria for iPSC. HEL24.3 cells were induced from a commercial fibroblast cell line CCD1112Sk (ATCC) obtained from the foreskin of a Caucasian neonatal male. This cell line has been previously characterized in (31) and has been submitted to the Human Pluripotent Stem Cell Registry using the identifier UHi006-A (https://hpscreg.eu/cell-line/UHi006-A). Patient iPS cell lines 137.2 and 138.6 were karyotyped using G-band analysis (300-400 bands/haploid chromosome number according to ISCN 2020 guidelines (32) with 20 mitoses analyzed per cell line. The karyotyping was performed and analyzed by the accredited HUSLAB laboratory, HUS Diagnostic Center, Helsinki, Finland (https://huslab.fi).

The APECED patient -derived iPS cells were confirmed to contain the homozygous R25X mutations by sequencing at the HUSLAB laboratory, HUS Diagnostic Center, Helsinki, Finland.

Relevant clinical and demographical information of the donors at time of donation are compiled in **Table 1**.

All iPS cells were cultured on Matrigel (Corning) -coated culture dishes using Essential 8 and Essential 8 Flex (Gibco) -media and passaged using 0,5 mM EDTA PBS (UltraPure 0.5M EDTA, Gibco; Phosphate Buffered Saline (1X) without Calcium and Magnesium, Lonza). All cells were kept in a humidified incubator at +37°C with 5% CO_2.

Generation of EBs

Self-aggregated EB suspensions were generated using a protocol modified from (33) and (34). On day 0, 80% confluent 6 cm iPSC culture dishes were rinsed with 0,5 mM EDTA, and then incubated 4 minutes in 0,5 mM EDTA. EDTA was aspirated, and colonies were harvested into a 6-well on Ultra Low Attachment plate (Corning Costar) with a 1:1 mix of Essential 8 media and DMEM (Dulbecco's Modified Eagle Medium, Lonza) with 20% heat-inactivated FBS (Fetal Bovine Serum, Gibco) + 1% GlutaMAX (Gibco) and supplemented with 1x RevitaCell (Gibco). The cells were cultured in a humidified incubator at +37°C with 5% CO_2. Next day (d1), media was replaced with EB growth media consisting of a 1:1 mix of DMEM + 10% FBS + 1% GlutaMAX and Essential 8 media. On days 2-4, 50-75% of the media was replaced daily. From day 5 onwards, 50-75% of the media was replaced every other weekday.

RT-qPCR

The iPS cell cultures and EBs harvested on days 3, 7 and 30 were lysed with QIAzol (Qiagen) and total RNA was isolated using a chloroform, isopropanol, and ethanol purification according to Qiagen's 'Lysis and Homogenization of Fatty Tissues Using the Tissueruptor' protocol followed by RNA cleanup using a RNeasy Mini Kit (Qiagen). Equal amounts of RNA per experiment were reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's protocol. Expression levels were detected using iQ SYBR Green Supermix (Bio-Rad) and run in a LightCycler 480 II (Roche) with conditions: 1x 95°C 5 min; 40x (95°C 18s, 57°C 20s, 72°C 20s) using primers listed in **Supplementary Table 1**. Relative fold change of mRNAs were analyzed using the $-2^{-\Delta\Delta Ct}$ method (35), with GAPDH1 used as a housekeeping gene.

Immunocytochemistry

For cytospin preparates, iPSCs were detached using TrypLE Express (Gibco), resuspended into cold PBS and cytocentrifuged onto glass slides. The slides were fixed with 10% formalin for 10 min at RT. Samples of EBs were collected on days 3, 7 and 30 of culturing and fixed with 10% formalin for 20 min at RT. The EB samples were then dehydrated through increasing concentration of EtOH and lastly xylene, embedded in paraffin and sectioned onto glass slides. The FFPE EB samples were first deparaffinized through a rehydration series of xylene and decreasing concentrates of EtOH. The EB and iPSC samples were heated in citrate buffer (pH 6.2) for antigen unmasking, washed with 0.05% Tween20 (Sigma Aldrich) in PBS and blocked with 2.5% normal horse serum for 30 min at RT. The samples were then incubated with primary antibodies (listed in **Supplementary Table 2**), diluted into 1% BSA (Sigma Aldrich) in PBS) o/n at +4°C, except for Ki-67 in iPSCs which was incubated 1h at RT.

An ImmPRESS Duet Double Staining Polymer Kit (Vector Laboratories) was used to demonstrate colocalization of AIRE + OCT4/NANOG. After primary antibody incubation, the samples were incubated with the secondary antibody for 30 min at RT and stained with ImmPACT DAB EqV substrate (Vector Laboratories, RRID: AB_2336521) and Permanent HRP Green Kit (Nordic BioSite) at RT.

For Ki-67 immunostaining, the secondary antibody Vector Universal ImmPRESS kit (Vector Laboratories, RRID: AB_2336534) and Vector NovaRED Substrate Kit, Peroxidase (HRP) (Vector Laboratories, RRID:AB_2336845) were used.

Apoptosis was quantified using the ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, RRID:AB_2661855) according to the manufacturer's protocol. The color was developed with Vector NovaRED Substrate Kit, Peroxidase (HRP) (Vector Laboratories, RRID:AB_2336845) at RT.

Lastly, samples were counterstained with Meyer hematoxylin, and after dehydration, overlaid with Pertex (Histolab) and covered with coverglass. Samples were imaged with a Leica DMLB microscope. The images were captured with a MicroPublisher RTV 5.0 camera (QImaging) using QCapture Pro 6.0 -software. Immunocytochemistry stainings were scored in blind by two individuals estimating the amount of positive cells on a scale of 0-33%, 34-66%. 67-99%, and > 99% cells per sample. Representative images of scorings are shown.

All statistical analyses were performed in IBM® SPSS® Statistics version 25. Results were analyzed using one-way ANOVA and Dunnett's t-tests.

RESULTS

APECED Patient-Derived iPSCs Do Not Have Any Major Proliferative Defects

APECED patient-derived PBMCs were induced to iPS cells with the widely used CytoTune system. During and upon induction, the pluripotent stem cells displayed normal induction

TABLE 1 | Relevant clinical and demographical information of the donors at time of blood/tissue donation for the generation iPSC.

iPS cell line	Age/gender of donor	APECED disease components	AIRE mutation
137	17/female	CMC, HPT, AD	homozygote R257X (c.769 C>T)
138	15/female	CMC, HPT, AD, TIN	homozygote R257X (c.769 C>T)
HEL24.3	neonate/male	N/A	none

CMC, chronic mucocutaneous candidiasis; HPT, hypoparathyroidism; AD, Addison's disease; TIN, tubulo-interstitial nephritis.

characteristics, morphology and formed iPSC colonies at expected efficiency (data not shown). Approximately 10 clones per patient were generated and two clones per patient selected for detailed experimental analysis. After passaging the APECED iPSC cells for 40+ passages, no discernible differences between the clones from any single patient have arisen. All clones exhibit a normal stem cell -associated morphology and display typical pluripotency characteristics: the cells are small and form tightly packed, round colonies with distinct borders and a high nucleus-to-cytosol ratio (30). Therefore, only one clone per patient was selected for detailed studies herein. Additionally, these two clones termed 137.2 and 138.6 were analyzed by standard Giemsa-band staining in a karyotyping assay showing both patient cell lines to have normal 46, XX karyotypes devoid of any structural or numerical chromosome abnormalities (data not shown, images available upon justified request).

As these cells had no discernible phenotypic or morphologic differences to the healthy control clone HEL24.3, we wanted to study their proliferative properties in more detail. Using immunocytochemistry, we analyzed the expression of nuclear protein Ki-67 (36) which is a widely used marker for proliferation. We found that APECED iPSCs display comparable proliferative capacity to healthy control iPSCs with no quantifiable difference in Ki-67 positivity (**Figure 1**), showing that APECED patient-derived iPSC do not have any proliferative defects.

APECED iPSCs Display Similar Stem Cell Marker Expression as Healthy Control iPSCs

As AIRE-deficient iPSCs have not previously been developed or characterized, we wanted to validate their pluripotency potential by studying whether these cells express the core pluripotency genes *OCT4*, *SOX2* and *NANOG* (37) as well as stem cell defining genes *TDGF1*, *p53*, *MYC*, *CD95/Fas* and *LIN28* (38–40).

Firstly, we examined by quantative real time PCR (qPCR) the mRNA expression of these markers in iPSCs. Expression of *p53*, *Myc* and *CD95/Fas* mRNAs was similar in both AIRE wild type and AIRE R257X iPSCs suggesting that loss of functional AIRE does not affect the aforementioned factors. Additionally, the expression of *LIN28A* and *LIN28B* were assessed and *LIN28B* showed an increase, though it was not statistically significant (**Figure 2**).

Next, protein expression and subcellular localization of AIRE, NANOG and OCT4 were examined using ICC on cytospin samples of APECED patient and healthy control iPSCs. The results of the AIRE and NANOG immunostaining are presented in **Figure 3**. No observable differences were found in the core stem cell markers' expression as the percentage of NANOG or OCT4 positive cells was comparable in the APECED iPSCs to the healthy control iPSCs. Next, we wanted to assess AIRE expression using an antibody raised against AIRE's N-terminal region. This antibody recognizes both the wild type as well as the truncated R257X mutant AIRE protein (41) and we found that both wild type HEL24.3 cells as well as patient iPSCs clones 137.2, and 138.6 expressed AIRE, however at very low levels with expression detected only in a few cells. Both the truncated AIRE in patient cells as well as the wild type AIRE in healthy person cell line were detected as a punctate pattern mostly in the cell nuclei but also in the cytoplasm as has been previously reported for AIRE (42, 43). Colocalization of AIRE and NANOG was detected in a few cells of healthy control HEL24.3 cell line, but not in APECED patient cell lines.

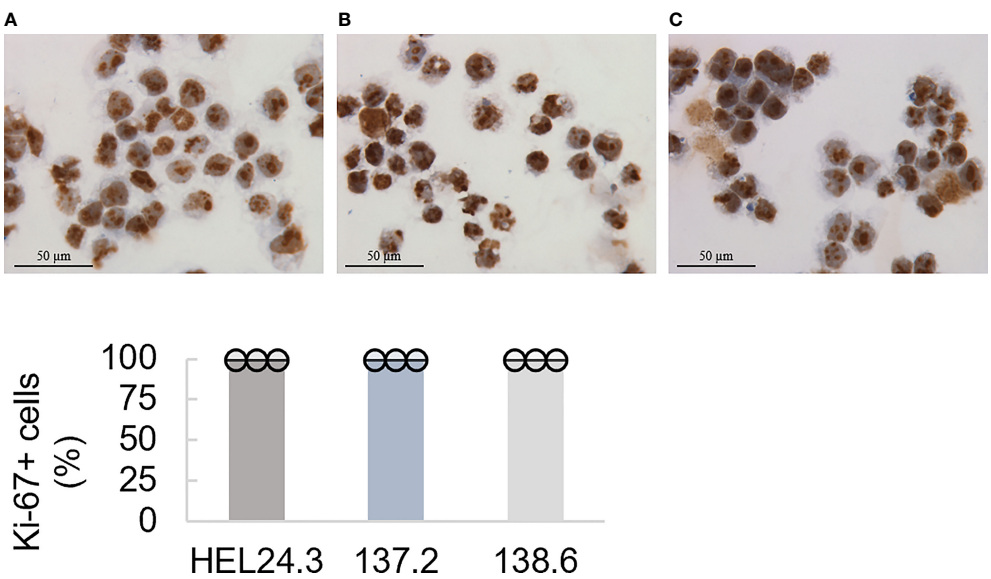

FIGURE 1 | Proliferation capacity of APECED patient-derived iPSCs. Top panel: The healthy control iPSCs HEL24.3 **(A)** and APECED iPSCs 137.2 **(B)** and 138.6 **(C)** display similar Ki-67 positivity. Bottom graph: Quantitation of proliferation (Ki-67+) in the iPSCs reveals they are similar irrespective of *AIRE* mutation. Dots represent three individually performed replicates, with error bars representing SD within a replicate. Bar graphs are the mean of three replicates, with error bars representing SEM of the replicates. No difference was observed between the cell lines.

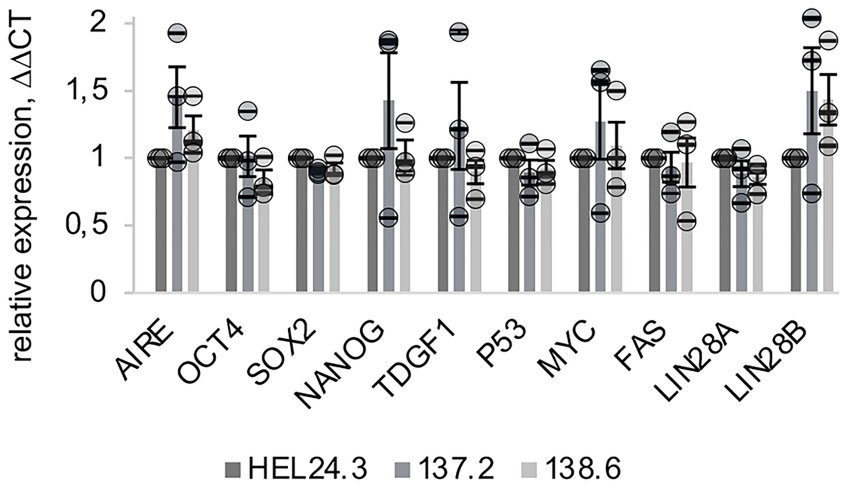

FIGURE 2 | Expression of AIRE and stemness-associated genes does not differ in APECED patient -derived iPSCs compared to healthy person-derived iPS cells. The relative mRNA expression of AIRE and common stem cell associated markers in APECED iPSCs (137.2 & 138.6) and a healthy control (HEL24.3) iPSCs. Expression levels presented as a mean of ΔΔCt fold change (columns) of three independently analyzed biological replicates (shown as circles), normalized to HEL24.3. Error bars of circles represent SD within a replicate. Error bars of columns represent SEM of the three replicates. No statistically significant difference was observed between the cell lines ($p > 0.05$).

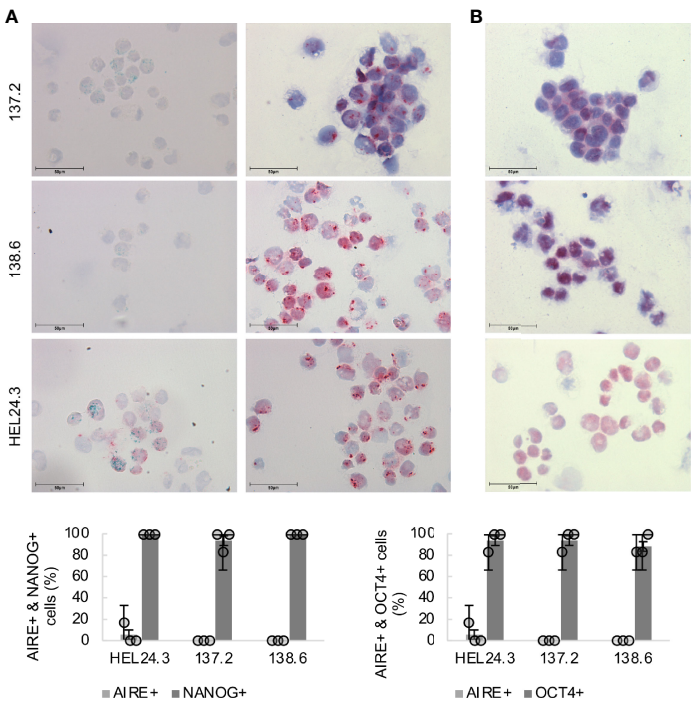

FIGURE 3 | Subcellular localization of AIRE and stemness-associated proteins does not differ in APECED patient -derived iPSCs compared to healthy person-derived iPS cells. Top panel **(A)**: AIRE (green) and NANOG (red) expression in iPSCs, **(B)**: OCT4 (red) in iPSCs. Mayer's hematoxylin (blue) used as nuclear counterstain. Bottom graph: Quantitation of AIRE+, NANOG+ and OCT4+ in healthy control iPSCs HEL24.3 and APECED iPSCs 137.2 and 138.6. Circles represent three individually performed replicates, with error bars representing SD within a replicate. The bar graphs represent the mean of the three replicates, with error bars representing SEM of the replicates. No statistically significant difference was observed between the cell lines ($p > 0.05$).

AIRE Deficient EBs Show No Gross Proliferative Defects

The ability to form EBs defines all pluripotent cells including iPSCs and they resemble the blastocyst phase of early embryos in their gene expression and epigenetic landscape (44). EBs also represent the onset of differentiation (45, 46) and thus we wanted to examine in detail whether mutated *AIRE* alters patient iPSC's pluripotency and early differentiation capacity. We generated EBs from patient and healthy control cells using the self-aggregation method (33, 34) observing the EB organoids for up to 100 days.

Early EBs were typically dense, solid spheroids but later the majority became cyst-like structures with projections emerging from the spheric central mass. To quantify the proliferative and apoptotic indexes of these EBs we chose to analyze their Ki-67 (36) and TUNEL activity (47). For this, we generated EBs from healthy HEL24.3 and APECED patient 137.2 and 138.6 clones, and harvested samples on days 3, 7, and 30. As shown in **Figure 4**, no discernible differences in proliferative nor apoptotic capacity was noted in APECED patient EBs. Indeed, all EBs had similar kinetics and distribution of apoptotic to proliferative cells. Furthermore, in all young EBs, the proliferative and apoptotic cells were typically evenly dispersed throughout the organoids. After day 7, the localization of proliferative and apoptotic cells became more dualistic, as Ki-67 was found especially on the cortical part of EBs, whereas apoptosis by TUNEL positivity was most prominent in the medullary regions (**Figure 4**). This reflects the classical event of clearance of the EB luminal cavity through caspase-dependent apoptosis (44).

AIRE and Stem Cell Marker Expression in APECED EBs is Similar to Healthy EBs

Next, we set out to quantify the mRNA expression of the core stem cell factors by qPCR in day 3, 7, and 30 aged EBs. The

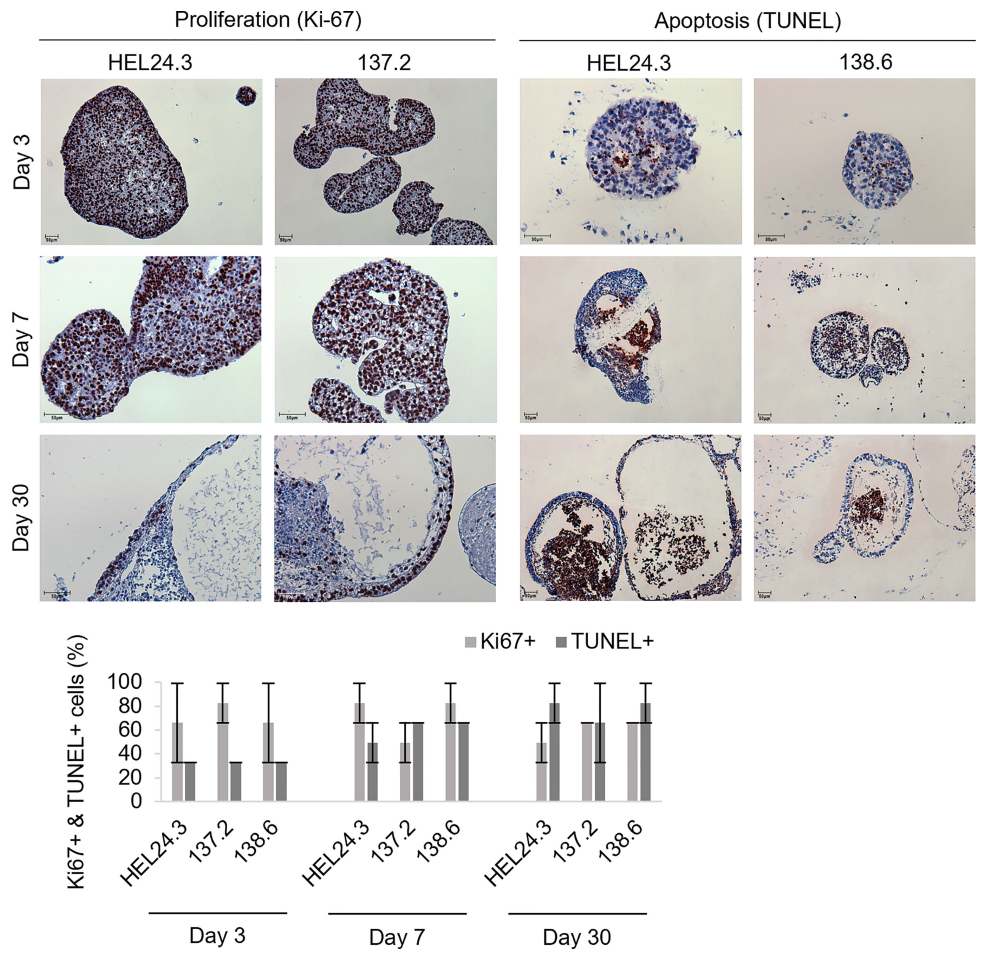

FIGURE 4 | Proliferation in APECED iPS-derived EBs is seen along the cortical layers and apoptosis within the medulla creating a lumen. Top panel: Proliferative (Ki-67, brown) and apoptotic (TUNEL, brown) cells in EBs generated from iPS cells of one healthy control HEL24.3 (first column and third column), and in two APECED iPS cell lines 137.2 (second column) and 138.6 (fourth column) as detected by Ki-67 and TUNEL immunocytochemistry, respectively. The FFPE samples were collected on days 3, 7 and 30 after generation of the EBs. Mayer's hematoxylin (blue) as nuclear counterstain. Bottom graph: Quantitation of proliferation (Ki-67 +) and apoptosis (TUNEL+) in the EBs reveals they are similar irrespective of AIRE mutation. Error bars represent SD.

results showed that *OCT4, NANOG, p53* and *TDGF1* mRNAs have similar kinetics and their expression decreased towards day 30 (**Figure 5**). This is consistent with published data where "ageing" of EBs correlates with decreased pluripotency marker expression (44).

We also set out to study stem cell marker protein localization within the EBs with immunocytochemistry. On day 3, EBs contained a marked number of OCT4 and NANOG-positive cells that were evenly dispersed throughout the individual EBs (**Figures 6A, B**), respectively). In day 7 and especially in day 30 EBs, the expression of NANOG and OCT4 became restricted to well defined areas of the outermost cortical layer. The number of positive cells and staining intensity of OCT4 and NANOG decreased as the EBs grew older. These data are in line with our qPCR data in **Figure 5** where both these factors' mRNAs decrease similarly. As for AIRE, the most intense staining was detected in the cortical layers of the EBs though some AIRE was also detected in the medulla and AIRE and NANOG/OCT4 stained positive in distinct cells and areas of the EBs (**Figure 6**).

DISCUSSION

In this study we wanted to explore a novel aspect of AIRE's potential role in induced pluripotent stem cells. We generated two iPSC lines from two female APECED patients and to our knowledge, this is the first study to generate and characterize APECED patient-derived iPSCs and to analyze the effects of their AIRE R257X truncation mutation. In addition to AIRE's pivotal role in establishing immune tolerance, recent studies have implicated AIRE in the self-renewal of ES cells, as Aire$^{-/-}$ ES cells have a significant impairment in their proliferation and organoid formation capacity. Additionally, another AIRE mutant was shown to induce mitotic defects during human reproduction and development (23, 26). Here we report initial data that APECED patient-derived iPSCs, harboring the AIRE R257X truncation mutation, have typical stem cell morphological features and display no impairment in proliferation as measured by Ki-67 positivity (**Figure 1**). The mutated AIRE protein was detectable by immunocytochemistry both in a nuclear (48) and a cytoplasmic, punctate pattern (49) as described before (42, 43). *AIRE* mRNA was detectable in very low quantities in iPSCs as shown by qPCR (**Figure 2**). Also, when we analyzed AIRE protein expression in iPSCs and EBs, only a small subset of cells stained positive. This expression pattern in pluripotent cells could reflect the stochastic and tightly controlled *AIRE* expression seen in thymic mTEC cells (18, 20). To our knowledge, there are no previous immunohistochemical analyses on AIRE protein abundance in pluripotent cells nor are there any assessments whether AIRE expression is limited only to a subset of stem cells or if it is more abundantly expressed. Based on our findings we propose that the functionally defective truncated AIRE does not seem disadvantageous to the overall proliferation capacity of iPSCs nor does it disrupt their ability to undergo the initial stages of differentiation as evidenced by EB formation.

Here, to validate the pluripotency of the APECED patient-derived iPSCs, expression of a set of stem cell factors and markers was assessed using qPCR. All iPSC clones independently of their AIRE mutation status expressed high levels of the pluripotency core genes, as well as stem cell associated markers *TDGF1* and *LIN28* (38) (**Figure 2**). This is in clear contradiction to previous data from animal models where Aire knockdown reduced the expression of Oct4 and Nanog in mouse ES (mES) cells thus decreasing their self-

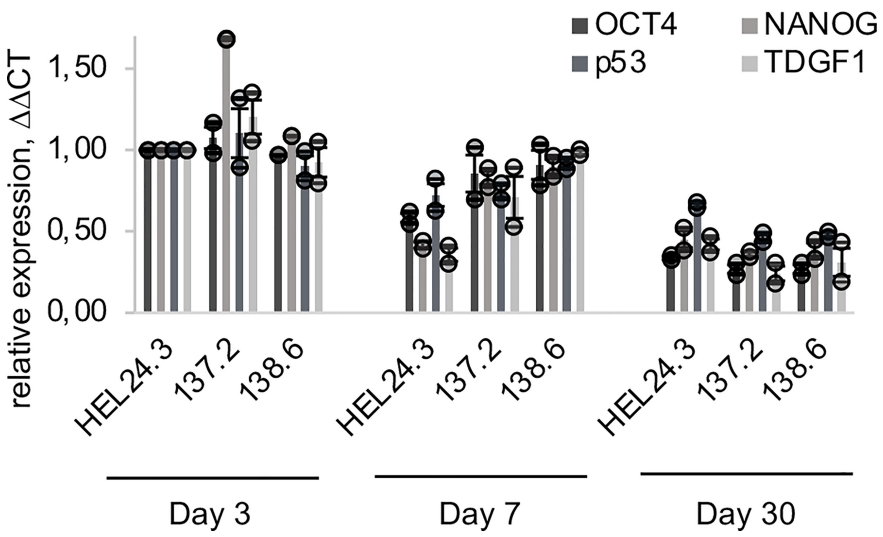

FIGURE 5 | qPCR quantification of *OCT4, NANOG*, *p53* and *TDGF1* expression in APECED and healthy person iPS-derived EBs. The relative mRNA expression in APECED EBs (137.2 and 138.6) and a healthy control (HEL24.3) EBs presented as a mean of ΔΔCt fold change of 2 independently analyzed biological replicates (shown as circles), normalized to the healthy control HEL24.3 day 3 sample. Error bars of the circles represent SD within a replicate. Error bars of the bars represent SEM of the two replicates.

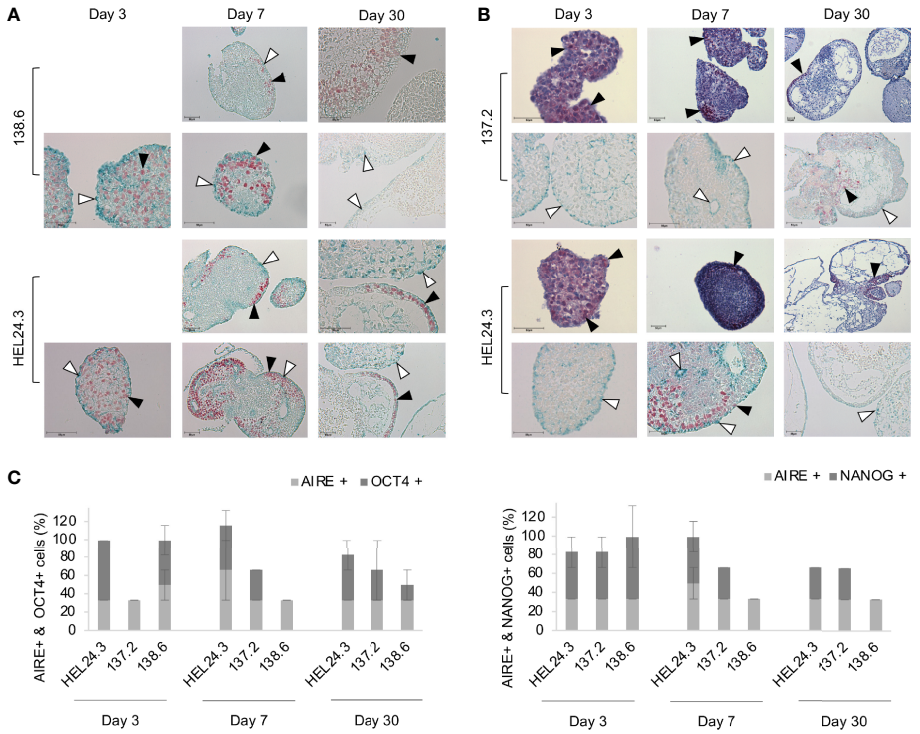

FIGURE 6 | AIRE mutation does not affect the spatial localization of AIRE, OCT4, or NANOG proteins in EBs. Panel **(A)** AIRE (green staining, indicated with white arrow heads) and OCT4 (red staining, indicated with black arrow heads) protein expression in EBs generated from iPS cells of APECED patient 138.6 and healthy control HEL24.3 on day 3, 7 and 30 after the generation of EBs. Panel **(A)** images are without Mayer's hematoxylin counterstaining. Panel **(B)** AIRE (green staining, indicated with white arrow heads) and NANOG (red staining, indicated with black arrow heads) protein expression detected by immunocytochemistry in EBs generated from iPS cells of APECED patient 137.2 and healthy control HEL24.3. FFPE samples collected on days 3, 7 and 30 after the generation. Mayer's hematoxylin (blue) used as a nuclear counterstain on images on the first and third rows. Images on second and fourth row are without Mayer's hematoxylin. Scale bar equals 50 µm. Panel **(C)** Quantification of positive cells in AIRE + OCT4 and AIRE + NANOG immunostainings shown in panels **(A, B)**. Error bars represent SD.

renewal capability (26). However, a possible explanation could be either iPSC heterogeneity (50) or that a full knockout of AIRE causes different biological outcomes compared to our R257X truncation mutant located in the SAND domain of AIRE. AIRE contains four major subdomains: the CARD/HSR, the SAND and two PHD finger- type zinc finger domains. The AIRE protein also contains four LXXLL domains that are found on coactivators of nuclear receptors (reviewed in (4)). Although the functions of these different domains of AIRE are established, the clinical outcomes of each individual AIRE mutation are still unknown. Currently over 100 AIRE mutation variants have been identified and only a subset have been exhaustively characterized for even their most fundamental clinical outcomes and associations. Also, as seen in APECED animal models, the manifestations of the AIRE mutation spectrum varies greatly depending on genetic background and species (4, 51). Thus it is entirely plausible, as our data suggest, that human AIRE-dependent pluripotency regulation could be more nuanced than in mouse models.

LIN28's two paralogues LIN28A and LIN28B are one of the classical stem cell markers and they regulate a complex network including OCT4, NANOG and SOX2 (25, 52). Aire has previously been shown to support the self-renewal of mouse ES cells through the activation of Lin28 and loss of AIRE lead to decreased expression of Lin28 (25). However, in this study the mRNA expression of *LIN28A* in APECED patient-derived iPSCs was only slightly decreased compared to healthy control iPSCs. This suggests that activation of LIN28A in humans might not be AIRE-dependent. However, we cannot exclude the possibility that this is due to the limited amount of iPSC clones we studied (one per patient) or the inherent heterogeneity in gene expression among iPS cell lines (50, 53–55). Also, CD95/FAS, p53 and MYC had comparable expression patterns both in heathy control HEL24.3 and APECED patient iPSCs clones 137.2 and 138.6.

The ability to form EBs is a quintessential feature of pluripotent stem cells enabling their propagation for extended periods of time (44). Thus, we chose to generate EBs to further investigate the stemness and early developmental phase properties of APECED patient derived iPSCs and to examine whether APECED EBs differ in stemness or proliferation. Satisfyingly, we detected no discernible differences in APECED EBs compared to healthy control EBs. The expression of OCT4 and NANOG, the core markers of pluripotency (37), decreases during differentiation (**Figure 5**) as expected (38, 56). We did not find colocalization of AIRE and NANOG or AIRE and OCT4, as

AIRE and NANOG/OCT4 protein immunostainings were found in distinct cells and areas of the EBs. In early EBs on day 3, OCT4 and NANOG expression was detected evenly throughout the organoids. Later the expression of NANOG and OCT4 became restricted to areas of the cortical layer and the number of positive cells decreased as the EBs grew older (**Figure 6**). This is in accordance with previous reports, as OCT4 expression is found in endoderm-like cells within the EB core and later during differentiation its expression is ultimately lost (44). Within early EBs, the proliferative Ki-67+ and apoptotic TUNEL+ cells were found evenly throughout the EBs but later their localization became almost mutually exclusive as Ki-67+ was found in the EB cortex and TUNEL+ on the edges and within the lumens. These results are in line with earlier reports showing young EBs as solid spheres which later evolve into cyst-shaped EBs with an external proliferative cell layer and internal apoptosis (57). No difference was detected between the healthy control and APECED EBs indicating that the R257X truncated AIRE does not seem to thwart normal proliferation of iPSCs.

Moreover, our results indicate that the most prevalent AIRE mutation (p.R257* located in the SAND domain) does not affect stem cell marker expression nor the pluripotency/EB formation of human iPSCs. This despite previous studies with Aire knockout mice have shown that both the mRNA and protein level of the core stem cell factors Oct4 and Nanog were decreased in $Aire^{-/-}$ ES cells and that the $Aire^{-/-}$ ES cells manifested impaired proliferation and colony formation (23, 26). One possible reason for these conflicting results might be that the truncated R257X AIRE in APECED patients could still be capable of performing some functions compared to the complete loss of AIRE in knockout animal models.

CONCLUSIONS

This study provides the first insights into APECED patient derived iPSCs and the effect of the most common underlying, disease-causing mutation in AIRE. Together these initial results indicate that the R257X mutation of the APECED patients does not abrogate pluripotency nor cause defects in proliferation, apoptosis, or the ability of these iPSCs to form EBs. These data open the possibility of generating iPSCs from APECED patients and using lentiviral gene-delivery to restore AIRE expression in these cells. While we did not attempt to differentiate the iPS cells we however postulate that possible differentiation into thymic precursors (27) or the keratinocyte lineage (58) could allow for the re-introduction of healthy autologous cells into APECED patients. Currently, we are commencing the restoration of keratinocytes in a rat model of APECED (51) where we will use a lentiviral vector constructed by us to restore endogenous expression of AIRE.

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 studies involving human participants were reviewed and approved by Helsinki and Uusimaa Hospital District (HUS) Ethics Committee of Medicine (HUS/1127/2016). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

Conceptualization of the study was done by KK, AH, and AR. Data curation, validation, visualization, methodology, and writing of the original draft were done by EK and AH. Moreover, EK, AH, and AR did the formal analysis and investigation of the study. The funding acquisition and project administration were supervised by AR and KK. AR was also in charge of the resources. The whole study was supervised, reviewed, and edited by AH and AR. 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/fendo.2022.794327/full#supplementary-material

Supplementary Table 1 | Primers used in qPCR

Supplementary Table 2 | Primary antibodies used for immunostaining. The monoclonal AIRE 6.1 antibody was produced in-house from a mouse hybridoma (41) and the clone was generated against a peptide comprising amino acids 1-206 of the N-terminus of the AIRE protein.

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Role of microRNA alternation in the pathogenesis of gouty arthritis

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Gouty arthritis is a common inflammatory disease. The condition is triggered by a disorder of uric acid metabolism, which causes urate deposition and gout flares. MicroRNAs are a class of conserved small non-coding RNAs that bind to the 3' untranslated region (UTR) of mRNA and regulate the expression of a variety of proteins at the post-transcriptional level. In recent years, attention has been focused on the role of miRNAs in various inflammatory diseases, including gouty arthritis. It is thought that miRNAs may regulate immune function and inflammatory responses, thereby influencing the onset and progression of the disease. This article mainly reviewed the roles of miRNAs in the pathogenesis of gouty arthritis and prospected their potential as diagnostic and prognostic relevant biomarkers and as possible therapeutic targets.

KEYWORDS

MicroRNA, gouty arthritis, hyperuricemia, cellular signaling pathway, biomarker

1 Introduction

Gouty arthritis (GA) is an inflammatory joint disease with a prevalence of 3.9% of all adults in the United States, 5.2% for men, and 2.7% for women (1). As a disorder of uric acid metabolism, this disease is mainly caused by the deposition of monosodium urate crystals (MSU) in the joint capsule, bursa, bone, and cartilage, ultimately causing joint damage and even deformity (2). With gout flares, the pain increases and seriously affects the patient's quality of life. In addition, gout is closely associated with metabolic comorbidities that can lead to myocardial infarction, type 2 diabetes, chronic kidney

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disease, and premature death (3, 4). The treatment of gouty arthritis attacks is mainly to control pain and suppress joint inflammation, such as the use of non-steroidal anti-inflammatory drugs, glucocorticoids, etc. The long-term management of patients with gouty arthritis focuses on uric acid-lowering therapy to reverse hyperuricemia and thus prevent gout attacks (5, 6). Despite new treatment strategies and a good understanding of the pathogenesis of gouty arthritis, recurrent attacks continue to occur after treatment (7).

MiRNA is a conserved short-stranded non-coding RNA of approximately 23nt in length, and they were first identified in Caenorhabditis elegans in 1993 (8). miRNAs are first transcribed in the nucleus as longer primary miRNAs (primary miRNA, pri-miRNA), then processed in the nucleus by Drosha into hairpin RNAs of 60-70 nucleotides, i.e. precursor miRNAs (miRNA precusor, pre-miRNA), which are transported out of the nucleus with the help of the Exprotin-5 complex and sheared in the cytoplasm by Dicer to become mature miRNAs (9, 10).Current studies have shown that miRNAs are highly conserved evolutionarily (11). A miRNA can regulate the activity or stability of multiple target genes by recognizing and inducing the assembly of the RNA silencing complex (RISC) with the miRNA response element (MRE) in the mRNA 3' untranslated region (UTR) region of the target gene, and multiple microRNAs can also synergistically regulate the same target gene (12, 13). More than 1,000 miRNAs have been identified in human cells, while more than 500 microRNAs in the human body (14). Although the functions of miRNAs are not fully understood, relevant studies have shown that miRNAs are involved in various processes, including cell differentiation, metabolism, and inflammation (15).

Recent research has demonstrated that miRNA plays an essential role in the pathogenesis of common nonautoimmune inflammatory diseases, including gouty arthritis (16). Although some studies have attempted to elucidate the crucial role of miRNAs in the pathogenesis of gouty arthritis, their analyses have always been conducted in a single direction. They have not diversified to integrate multiple fields of study. Therefore, this paper reviews the various regulatory mechanisms of miRNAs in developing gout, including its relationship with uric acid metabolism, classical inflammatory signaling pathways, and bone erosion. On this foundation, we considered the promise of miRNA as a potential diagnostic and prognostic marker for gout and as a therapeutic target.

2 Overview of gouty arthritis

Gouty arthritis (GA) is characterized by swelling and heat pain in one side of the joint, causing joint dysfunction, deformity, and even disability (17). Epidemiology reports the current range of gout incidence at 0.6-2.8 per 1000 people per year. The prevalence of gout has continued to increase worldwide in recent decades, probably due to the aging of society's population and changes in dietary patterns (18–20). The development of gout is based on four pathophysiological stages, the first two of which are hyperuricemia and the formation and deposition of sodium urate crystals, the leading causes of which are disorders of purine metabolism and dysregulation uric acid secretion (Figure 1). The latter two components are mainly gout flares triggered by acute inflammatory reactions and irreversible bone erosion caused by the deposition of tophi in advanced gout (21).

Usually, the intake of purines is converted by xanthine oxidase in the liver to uric acid, which is excreted mainly through the kidneys (22). The renal tubular transporters such as OAT4 and GLUT9 are responsible for the reabsorption of uric acid (23, 24), while ABCG2 and ABCC4 are responsible for the secretion of uric acid (25, 26). And once a high purine diet such as alcohol, seafood, and meat is accompanied by impaired renal excretion function, it will lead to hyperuricemia. Once a high purine diet is accompanied by inadequate excretion by the kidneys, it can lead to hyperuricemia (27, 28).

Due to the high concentration of urate, along with other physical and chemical environments, MSU is generated and deposited in the joint cavity, which in turn acts as foreign bodies to recruit neutrophils and macrophages and initiate toll-like receptor (TLR) and NLRP3 inflammasome signaling pathways to activate innate immunity (29–31). Current studies suggest

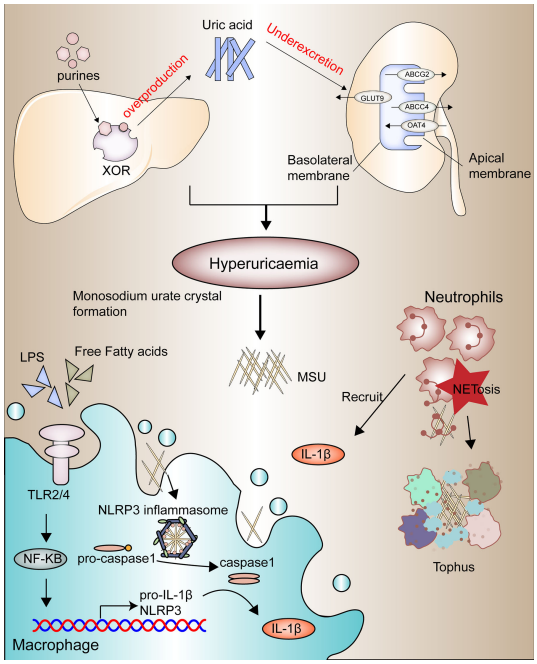

FIGURE 1
Four stages in the pathophysiological development of gouty arthritis.

that the acute flares of gout depend on two switches, one of which is the activation of the TLR2/4-NF-κB signaling pathway within macrophages or monocytes, which promotes the synthesis of pro-IL1-β and significant components of the inflammasome, and that this activation is associated with the influence of large amounts of free fatty acids, intestinal flora or other microorganisms (32, 33). Stimulating sodium urate crystals is another critical point of activation of gout inflammation, which activates the NLRP3 inflammatory pathway by promoting the assembly of inflammatory vesicles, thereby promoting the conversion of pro-caspase-1 to caspase-1 and the release of large amounts of pro-inflammatory factors such as IL-1β (34–36).

These pro-inflammatory factors recruit more neutrophils to exacerbate the inflammatory response; however, along with the inflammatory death of large numbers of neutrophils, activated neutrophils release extracellularly depolymerized chromatin and intracellular granule proteins, called neutrophil extracellular traps (NETs), to trap and break down inflammatory factors to relieve gout flares (37, 38). This chronic inflammatory response develops into the advanced disease characterized by tophi (Figure 1), a microenvironment of adaptive and innate immune cells, MSU, and fibroblasts, promoting bone resorption by osteoclasts and reducing bone formation osteoblasts, and ultimately causes bone erosion (39, 40).

3 The function and mechanism of miRNAs in GA

Extensive studies illustrate that abnormal expression in miRNAs occurs during the pathophysiology of gouty arthritis (15, 41, 42). Only 10% of the population with high uric acid has positive signs of gouty arthritis, which may be associated with different genetic backgrounds, and miRNA sequence alterations affect the genetic susceptibility background (43). In addition to this, human genome-wide association studies (GWAS) have identified many loci associated with hyperuricemia and gout, and these single nucleotide polymorphism (SNP) loci are mainly associated with the coding of uric acid transporter genes (44, 45). Further studies have illustrated the ability of miRNAs to regulate inflammatory immune-related processes in gouty arthritis (46, 47).

Over the past years, attempts have been made to identify aberrantly expressed MiRNAs in gout to explore the role of these molecules in the pathogenesis of gout. In 2014 Tae-Jong Kim et al. first investigated the role of Mir-155 in acute gouty arthritis (48). So far, studies on gouty arthritis have been divided into omics-based high-throughput studies and *in vitro* cellular models stimulated with MSU or *in vivo* animal models. A large number of meaningful results have now been identified. Therefore, we will summarize the relationship between miRNAs and gout pathogenesis in terms of hyperuricemia, inflammatory immunity, and bone erosion and look at their diagnostic and therapeutic value based on the last five years of publication and older but more classic literature

3.1 Involvement of miRNAs in the hyperuricemia

Hyperuricemia (HUA) is the prodromal stage of gout attack and a common clinical feature in the course of gouty arthritis. The inability to excrete uric acid from purines promptly leads to a series of disturbances in the metabolic environment and can even cause damage to liver and kidney function (49). As the body's primary urate handling organ, the kidney generally relies on renal tubular urate transport proteins, such as URAT1, GLUT9, and ABCG2, to regulate uric acid excretion (26, 44). It was reported that C421A polymorphism enhanced the degradation of ABCG2 in a miRNA-dependent manner and that the use of inhibitors of miR-519c and miR-328 reversed this translational repression (50). Sun, W. et al. reported that Xie-Zhuo-Chu-Bi-Fang could upregulate miR-34a and downregulate URAT1 to treat hyperuricemia (51). In addition, miR-143-3p can directly target the 3'UTR of GLUT9 in renal tubular epithelial cells to reduce uric acid reabsorption and inflammatory response (52). In a clinical study, miR-155 was elevated in the serum of HUA patients, and subjects with urate deposition had higher miR-155 than those without deposition findings (53). *In vitro* experiments also revealed that miR-155 was elevated in high-uric acid-stimulated venous endothelial cells (HUVEC) and inhibited eNOS expression causing endothelial cell dysfunction (54).

Similarly, hyperuricemic stimulation led to the downregulation of miR-92a, thereby inhibiting vascular neogenesis through the KLF2-VEGFA axis (55). Hong Q et al. also found that miR-663 could act on the transcript of TGF-β1 to regulate PTEN to inhibit endothelial cell migration (56). These studies also suggest that high uric acid causes cardiovascular damage and explains the correlation between gout and cardiovascular disease such as myocardial infarction. And miRNAs also play a precise regulatory role in liver and kidney function damage caused by excessive uric acid. Uric acid damages renal interstitial fibroblasts by downregulating miR-9 and causing activation of NF-KB and JAK-STAT pathways (57). Besides, Chen, S et al. also reported that overexpression of miR149-5p inhibited FGF21 expression and attenuated uric acid-induced lipid deposition in hepatocytes (58). Chi, K et al. found that HOTAIR competitively binds miR-22 in hyperuricemia to regulate NLRP3 inflammasome activation to promote endothelial cell pyroptosis and exacerbate renal injury (59). Recent studies have also reported a decrease in miR-30b and an increase in IL-6R in serum urine and kidney tissue in a mouse model of HUA (60). The above study we summarized in the Table 1. In summary, miRNAs are involved in the development of

TABLE 1 The miRNAs involved in Hyperuricemia.

miRNA	Result	model	reference
miR-22-3p↓	NLRP3 inflammasome↑, pyroptosis↑	*In vivo* and *in vitro*	(59)
miR-30b↓	IL-6R↑	*In vivo*	(60)
miR-34a↑	URAT1↓	*in vivo*	(51)
miR-149-5p↑	FGF21↓, hepatocytes lipid accumulation↑	*In vitro*	(58)
miR-143-3p↓	GLUT9↑	*In vitro*	(52)
miR-9↑	NF-KB↓, JAK-STAT↓, injury in NRK-49F↓	*In vitro*	(57)
miR-92a↓	KLF2↑, VEGFA ↓, Angiogenesis↓	*In vitro*	(55)
miR-663↑	TGF-β1↓, PTEN↓, migration↓	*In vitro*	(56)
miR-155↑	eNOS↓, endothelial dysfunction	*In vitro*	(54)

a "↑" indicates elevated expression or facilitation.
b "↓" indicates reduced expression or inhibitory effect.

hyperuricemia and play an important role, and targeting miRNA processing may provide new insights for the future treatment of hyperuricemia.

3.2 Role of miRNAs in the regulation of immune-inflammatory responses

3.2.1 miRNAs and TLR2/4/MyD88/NF-KB pathway in GA

The onset of gouty arthritis results from an inflammatory immune response triggered by MSU deposition. Two pathways mediate the main molecular mechanisms: activation of the TLR-related NF/KB signaling pathway and activation of the inflammatory vesicle NLRP3, respectively. The former is mainly microbial or free fatty acids activating Toll-like pattern recognition receptors, mainly TLR2/4, recognizing the downstream signaling molecule myeloid differentiation factor 88 (MyD88) for intracellular signaling and finally leading to the activation of NF-KB (31, 35, 61). Numerous studies have illustrated the significant correlation between miR-192 and NF-KB pathway. For example, miR-192-5p effectively alleviated tumor progression by inhibiting the IRAK1/NF-κB pathway in endometrial cancer (62, 63). Recently, Lian, C et al. found that miR-192-5p in MSU-treated synovial fluid mononuclear cells (SMFCs) and THP-1 could target TLR4 to inhibit NF-KB pathway activation reducing TNF-a and IL-1β release (Figure 2) (64). MiR-146a is the first regulator that is involved in innate immunity. It has been reported that miR-146a can regulate key downstream adaptor molecules of TLR in sepsis by complementary pairing with the 3'UTR base sequence of TNF receptor-associated

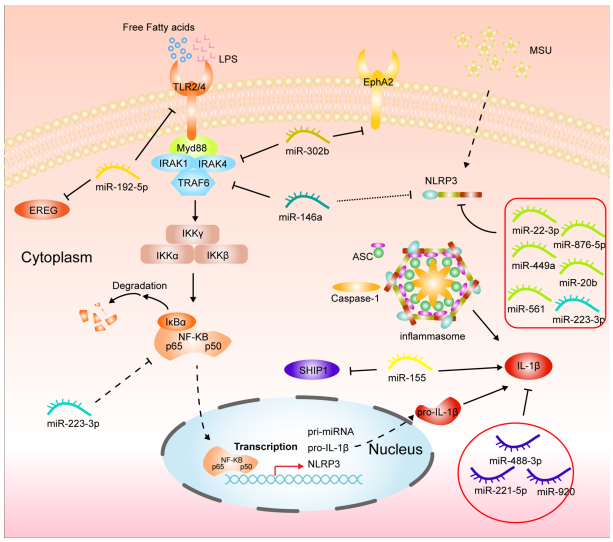

FIGURE 2
Mechanisms of miRNAs in gouty arthritis regulating inflammatory pathways in monocytes/macrophages.

factor 6 (TRAF6) and IL-1 receptor-associated kinase 1 (IRAK1) genes, thereby inhibiting the activity of TLR signaling pathway and thus inhibiting NF- κB signaling pathway to exert inflammatory suppressive effects (65). Another study indicates miR-146a alleviates inflammation in acute gouty arthritis in rats *via* TLR4/MyD88/NF-KB signaling pathway. And further study demonstrated that miR-146a knockout mice promoted the development of gouty arthritis by upregulating TRAF6 and IRAK-1 expression compared to wild type (Figure 2) (66, 67). Ma, T et al. illustrated that MicroRNA-302b could directly bind to the 3' UTR of IRAK4 and EphA2 in an *in vivo* and *in vitro* model of gout to inhibit activation of the NF-KB pathway to reduce IL-1β (Figure 2) (68). Therefore, targeting the TLR-mediated NF-KB pathway *via* miRNA can be a promising approach for GA treatment.

3.2.2 miRNAs and NLRP3 inflammasome pathway in GA

NLRP3 is the most comprehensive subfamily studied in the nucleotide-binding oligomerization domain-like receptor (69). NLRP3 inflammasome comprises NLRP3, recruitment domain, an adaptor protein, apoptosis-associated spot like protein (ASC), and caspase-1, expressed in many immune cells. In gouty arthritis, MSU crystal acts as a ligand to bind and activate NLRP3 inflammasome. After the conformational change of NLRP3 protein, it polymerizes with ATP to form a protein oligomer. It then recruits pro-caspase-1 and ASC through its effector domain PYD, The caspase-1 precursor was automatically catalyzed into the active form, and proIL-1β was enzymolysis into IL-1β (70, 71). Current studies have confirmed that miRNAs are critical regulators of the NLRP3 inflammasome pathway (72). In recent years, it has been reported that circHIPK3 can act as a molecular sponge to adsorb miR-561 to competitively bind NLRP3 mRNA to reduce inflammation in GA (64). Upregulation of miR-20b expression and downregulation of NLRP3 protein was also found in macrophages with HOTAIR knockdown (73). Wang, X et al. reported that miR-223-3p and miR-22-3p could reduce inflammatory effects in monocytes and mouse models of gout by interacting with the 3' untranslated region segment of NLRP3 mRNA (74). And Tian, J et al. also found that miR-223 could target NLRP3 mRNA in MSU-induced rat models and fibroblast-like synoviocytes to inhibit inflammation and cellular pyroptosis. Further studies confirmed that miR-223 deficiency exacerbated the swelling index of MSU-induced joint inflammation and intensified inflammatory cell infiltration and cytokine release, including IL-1β and MCP-1, compared to WT mice (75, 76). Similarly, the expression of NLRP3 was dramatically upregulated in Bone marrow-derived macrophages (BMDMs) from miR-146a KO mice (67). Besides, the active ingredients of some Chinese traditional medicines also exert anti-inflammatory effects in GA by regulating the miRNA/NLRP3 axis. Total glucosides of paeony alleviate *in vitro* MSU-induced inflammation in macrophage THP-1 *via* the MALAT1/miR-876-5p/NLRP3 cascade pathway. Wang Y et al. also reported that Tripterine alleviates GA by modulating miR-449a to act directly on NLRP3 mRNA and inhibit its expression (Figure 2) (77, 78).

3.2.3 miRNAs and other mechanisms of inflammation in GA

In GA, when inflammatory pathways in macrophages are activated, they are polarized toward the M1 phenotype and release large amounts of pro-inflammatory factors such as TNF-a, IL-1β, and MCP-1. miRNAs also play a regulatory role in this process. MiR-449a and miR-192-5p can target NLRP3 and epiregulin (EREG) to inhibit macrophage M1 polarization in gout (77, 79). Apart from this, miRNA-488-3p and -920 both can interact with the 3'UTR of IL1-β to exert anti-inflammatory effects (80). Li, G. et al. also reported that miR-221-5p represses IL1-β expression in acute gouty arthritis to regulate the inflammatory response (81). Although Jin, H. M et al. reported that overexpression of miR-155 *in vitro* reduced SHIP-1 levels and promoted MSU-induced TNF-a and IL-1β production (Figure 2), it was later indicated that there was no remission of GA in miR-155 knockout mice compared to wild type (48, 82). Inflammatory factors tend to infiltrating immune cells, such as neutrophils, which on the one hand, worsen inflammation.

On the other hand, large numbers of neutrophils accumulate inflammatory death, chromatin remodeling, and ejection outside the cells to form NETs, called NETosis. These aggregated NETs can trap pro-inflammatory factors and act in conjunction with some anti-inflammatory factors to reduce the development of inflammation. Yet, few studies on the relationship between miRNAs and NETs in gout. Recently, it has been shown that MSU stimulation can significantly increase miR-3146 expression in neutrophils, accompanied by the formation of many NETs. In contrast, treating rats with antagomir-3146 reduced NETs formation and relief of joint swelling and inflammation, suggesting that early NETs formation exacerbates GA and that miR-3146 plays a role vital role before NETs formation (83). However, the current research on NETs is still inadequate, and how miRNAs regulate the development of NETs in gout is still unclear. Further studies are needed to reveal the potential mechanisms to help people understand gout and identify potential therapeutic targets. We conclude with a summary of miRNAs involved in inflammatory immunity. (Table 2)

3.3 The regulatory role of miRNAs in bone erosion

There is irreversible joint damage and deformity in advanced gout, mainly due to local cartilage damage and bone erosion caused by tophi (40). The primary mechanism is that MSU

TABLE 2 The miRNAs involved in inflammatory immunity.

miRNA	target gene/pathway	Role in GA	model	reference
miR-223-3p	NLRP3/NF-KB	–	*In vivo* and *in vitro*	(74–76)
miR-449a	NLRP3	–	*In vivo* and *in vitro*	(77)
miR-22-3p	NLRP3	–	*In vivo* and *in vitro*	(74)
miR-3146	SIRT1	+	*In vivo* and *in vitro*	(83)
miR-876-5p	NLRP3	–	*In vitro*	(78)
miR-20b	NLRP3	–	*In vitro*	(73)
miR-561	NLRP3	–	*In vitro*	(64)
miR-192-5p	TLR4 EREG	–	*In vivo* and *in vitro*	(64, 79)
miR-221-5p	IL1-β	–	*In vitro*	(81)
miR-146a	TLR4/MyD88/NF-KB TRAF6/IRAK1/NLRP3	–	*In vivo*	(66, 67)
miR-155	SHIP-1	+	*In vivo* and *in vitro*	(48, 82)
miR-302b	IRAK4 EphA2	–	*In vivo* and *in vitro*	(68)
miR-488-3p	IL1-β	–	*In vitro*	(80)
miR-920	IL1-β	–	*In vitro*	(80)

a "-" indicates an inhibitory role during disease progression.
b "+" indicates a promoting role during disease progression.

disrupts the balance between osteoblasts for bone formation and osteoclasts for bone resorption, decreases the activity of osteoblasts, promotes the aggregation and differentiation of osteoclasts, and promotes the development of inflammation and bone damage (84–86). Extensive studies have confirmed the involvement of miRNAs in the development of bone erosion. For instance, miR-20a targets RANKL through the TLR4/p38 pathway, hindering osteoclast proliferation and differentiation (87). Sujitha, S et al. found that miR-23a altered the expression level of LRP5 through RNA interference and contributed to a decrease in bone loss and an increase in calcium retention (88). In addition, Najm, A. et al. also demonstrated that miR-17 inhibits the autocrine effects of the IL-6 family *in vivo* by directly targeting JAK1 and STAT3 to exert anti-inflammatory and anti-bone erosion effects (89). There are few studies on miRNAs affecting bone erosion in gouty arthritis. Only An L et al. reported that miR-192-5p could inhibit MSU-induced EREG expression in GA mice to alleviate bone erosion (79). This case suggests that the underlying molecular mechanisms of miRNAs affecting bone erosion in GA remain to be explored.

4 Application of miRNAs in clinical diagnosis and treatment of GA

First of all, miRNA widely exists in a variety of body fluids, such as whole human blood (90), urine (91) saliva (92). Secondly, miRNA is stable in body fluid in a specific secretion mode, and it is easy to extract tissue samples without invasion. Even under changing environmental conditions, miRNA can stably exist. Because of its specificity, sensitivity, and stable expression in a wide range of diseases, MiRNA has early diagnostic capabilities and is rapid and accurate (92). In studies of gouty arthritis, several miRNAs are up-or down-regulated, and some of these miRNAs also vary with the extent of the disease. BohatáJ et al. found elevated levels of five circulating miRNAs, miR-17, miR-18a, miR-30c, miR-142, and miR-223, in the plasma of patients with GA and HUA (93). In addition, it has been reported that miR-221 is lowly expressed in the serum of AGA patients and the receiver operating curve (ROC) applied to the diagnostic value analysis showed an area under the curve of 0.884 (81). Therefore miRNAs have the potential to become markers for gout diagnosis.

The treatment of GA is mainly divided into anti-inflammatory and analgesic, and uric acid lowering. The primary treatment for gout attacks is cortisol, non-steroidal anti-inflammatory drugs (including selective and non-selective COX2 inhibitors), and low-dose colchicine to control pain and lessen inflammation (94–97). Although IL-1 inhibitors can effectively control gout attacks, they are usually reserved for patients with intolerable side effects or contraindications to first-line anti-inflammatory therapy (98, 99). The first-line uric acid-lowering therapy drug is allopurinol, a xanthine oxidase inhibitor. Still, patients who do not respond to or are intolerant of allopurinol are treated with febuxostat (6, 100). Probenecid, sulfinpyrazone, and benzbromarone can be used as monotherapy or combined with xanthine oxidase inhibitors by promoting uric acid excretion (101, 102). Liu, P et al. found that colchicine upregulated mir-223-3p and downregulated IL-1β, and etoricoxib treated AGA by upregulating miR-451a and downregulating COX-2 (103). A recent study reported that

two novel hexapeptides (GPAGPR and GPSGRP) found in Apostichopus japonicus hydrolysates inhibit uric acid biosynthesis and reabsorption. The expression profiles of GPSGRP-treated HUA model mice were analyzed, and 21 differentially expressed miRNAs were identified (104).

Chinese medicine or natural products have been developed to treat gout arthritis in recent years. The drugs for acute gout arthritis have severe adverse reactions such as bone marrow suppression, liver cell damage, and gastrointestinal bleeding (105). Traditional Chinese medicine has some advantages in terms of low toxicity and adverse reactions. Wang Y et al. indicated that both Chuan Hu Tong Feng Compound and Allopurinol upregulated miR-486-5p, miR-339-5p, and miR-361-5p expression and decreased CCL2 and CXCL8 protein levels in patients with chronic gouty arthritis (106). Another research proved that benzbromarone and Xiezhuo Chubi Decoction reduced uric acid levels by increasing the expression levels of miR-34a and miR-146a (107). In addition, like Tripterine (77), Total glucosides of paeony (78) and Epigallocatechin (57) were also reported to regulate the expression of miR-449a, miR-876, and miR-9, respectively, to alleviate the progression of GA. Similarly, Li, X et al. reported that Noni (Morinda citrifolia L.) fruit Juice also modulates miRNA and pro-inflammatory factors to treat MSU-induced AGA in mice (108).

Although miRNA has much fundamental research on the treatment of arthritis, there are still many problems in the transition from mechanism research to clinical application. Therefore, miRNAs related to gout treatment need further exploration and development.

5 Future expectation

Since the discovery of miRNAs, their wide range of biological effects have been gradually revealed, also indicating that they play an important regulatory role in various cellular activities. Based on recent research results in related fields, in this section, we will look at the future directions of miRNA research and the prospects of clinical applications.

Exosomes, a cellular vesicle structure widely found in body fluids, have been identified for the presence of miRNAs. In recent years, several studies have demonstrated that miRNAs regulate inflammatory immunity and tumor progression through exosomes as vectors. Jiang, K et al. found that peripheral-derived exosome-mediated miR-155 promoted the polarization and proliferation of macrophage M1 and activated the NF-KB pathway to promote the release of inflammatory factors TNF-α and IL-6 in an acute lung injury model in mice (109). Similarly, Yingying Cao et al. reported that Enterotoxigenic Bacteroides fragilis (ETBF) promotes intestinal inflammation and malignancy by inhibiting exosome-encapsulated miR-149 (110). Naïve bone marrow-derived macrophages produce exosomes with anti-inflammatory miRNAs that target receptor macrophages to promote their M2 polarization and alleviate inflammation (111). Therefore, it is natural to speculate that circulating exosomes contain miRNAs that may influence the development of GA by regulating macrophage polarization or other key molecules of inflammation. Furthermore, such exosome-derived miRNAs have great application in both the diagnosis and treatment of GA.

In addition, nanomaterials as carriers of drug-targeted delivery systems have become a hot research topic due to the development of the interdisciplinary intersection of materials science and medicine in recent years. Since miRNA mimics are not resistant to nucleic acid endonucleases and are prone to degradation in circulation, nanomaterials can be used to wrap miRNAs for targeted therapy. Wang, F et al. reported that microRNA-31 bound to adriamycin-loaded mesoporous silica nanoparticles would be used to target tumor cells high in MTEF4 expression to promote mitochondrial apoptosis (112). Moreover, Ahir, M. et al. also reported that the use of mesoporous silica nanoparticles as co-delivery carriers of miR-34a-Mimic and antisense-miR-10b on tumor cells effectively inhibited tumor growth and metastasis in triple-negative breast cancer (113). Although nanomaterials are being studied as potential therapies for tumors and inflammatory diseases, they may still cause an immune response in the body and exacerbate inflammation. Therefore the development of low inflammatory response nanomaterials for the treatment of inflammatory diseases remains a great challenge. With the development of basic science and technology, gene-editing technology is becoming more and more mature and is expected to be used for the treatment of many diseases. CRISPR/Cas9 technology, which won the Nobel Prize, has brought a revolution to the life science field. Recently, Zhou, W et al. reported that CRISPR/Cas9-mediated knockdown of miRNA-363 effectively promoted apoptosis in diffuse large B-cell lymphoma cell lines in response to adriamycin-induced apoptosis (114). And, Yu Toyoda et al. also utilized CRISPR/cas9 to construct knockout mice to identify the role of GLUT12 in regulating blood urate levels (115). Therefore, the use of CRISPR/cas9-mediated miRNA knockdown to suppress inflammation in gouty arthritis still holds great promise for research.

6 Conclusion

In recent years, miRNAs have become a hot topic in biomedical research. Current studies have shown that miRNAs are closely associated with the development and progression of gouty arthritis, and miRNAs play an essential role in the post-transcriptional regulation process of genes. Despite the large number of studies reporting miRNA regulation of the gouty inflammatory process, there is still a significant gap in gouty arthritis, especially in bone erosion.

The underlying mechanisms of self-remission of gout as a recurrent chronic disease and the formation of gouty stones remain unclear. What is neutrophil-associated NETosis in the inflammatory process, and is it the culprit that exacerbates inflammation or relieves it leading to recurrent gout attacks?

Although gout is already a treatable rheumatic disease, the side effects of drugs are still evident, and targeting miRNAs may provide a new idea and insight for gout treatment. In the future, miRNA is expected to be a marker for diagnosing gouty arthritis or a target for drug therapy. However, further studies are still needed. Therefore, the search for relevant miRNAs and further study of their mechanisms are essential for diagnosing and treating gouty arthritis.

Author contributions

TX and JWu conceived and designed the article, ZL and FY wrote the manuscript, SH and JWa reviewed the literature, BC, LL and JY revised the article, and YY, CY, YH, and SW proofread the language. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Role of irisin in physiology and pathology

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Irisin, out-membrane part of fibronectin type III domain–containing 5 protein (FNDC5), was activated by Peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1α (PGC-1α) during physical exercise in skeletal muscle tissues. Most studies have reported that the concentration of irisin is highly associated with health status. For instance, the level of irisin is significantly lower in patients with obesity, osteoporosis/fractures, muscle atrophy, Alzheimer's disease, and cardiovascular diseases (CVDs) but higher in patients with cancer. Irisin can bind to its receptor integrin αV/β5 to induce browning of white fat, maintain glucose stability, keep bone homeostasis, and alleviate cardiac injury. However, it is unclear whether it works by directly binding to its receptors to regulate muscle regeneration, promote neurogenesis, keep liver glucose homeostasis, and inhibit cancer development. Supplementation of recombinant irisin or exercise-activated irisin might be a successful strategy to fight obesity, osteoporosis, muscle atrophy, liver injury, and CVDs in one go. Here, we summarize the publications of FNDC5/irisin from PubMed/Medline, Scopus, and Web of Science until March 2022, and we review the role of FNDC5/irisin in physiology and pathology.

KEYWORDS

irisin, beige fat, musculoskeletal homeostasis, cancer, liver, cardiovascular diseases

Abbreviations: UCP-1, uncoupling protein 1; Dio2, type II iodothyronine deiodinase 2; Cidea, cell death activator; Prdm16, PR domain-containing 16; Cox-7a, cytochrome c oxidase subunit 7a; PINK1, PTEN-induced putative kinase 1; RANKL, receptor activator for nuclear factor–κB ligand; RANK, receptor activator of nuclear factor–κB; NFATc1, nuclear factor of activated T cell 1; TRAP, tartrate-resistant acid phosphatase; CK, cathepsin K; IGF1, insulin-like growth factor 1; FOXO1, forkhead box protein O1; SOST, Sclerostin.

1 Introduction

The protein sequence of fibronectin type III domain–containing 5 protein (FNDC5) contains a signal peptide [for endoplasmic reticulum (ER) targeting nascent FNDC5] (1), a hydrophobic transmembrane domain, a fibronectin III domain (the main part of irisin in the extracellular), and a carboxyterminal domain in the cytoplasm. After being N-glycosylated at the two potential sites—Asn36 and Asn81 (mouse) (2) or Asn7 and Asn52 (human) (3) in the ER—and cleaved by disintegrin and metallopeptidase domain (ADAM) family proteins such as ADAM10 (4), irisin is secreted to the blood circulation.

The physiological role of irisin in inducing thermogenic beige fat genesis to control energy metabolism was first described by Spiegelman and his teammates in 2012 in *Nature* (5). After that, irisin has also been found to promote liver glycogen synthesis and inhibit liver gluconeogenesis to maintain glucose homeostasis (6, 7). Later, the function of irisin in nerve system was found in improving cognition, learning, and memory (8). Moreover, irisin also contributes to maintaining musculoskeletal homeostasis by binding with integrin αVβ5 (9, 10). In recent years, researchers have also revealed that irisin reduces the risk of cancers (11) and cardiovascular diseases (CVDs) (12).

In this review, we summarized the up-to-date publications on irisin. We hope that it will help to understand the mechanisms of irisin and provide clues for the clinical application of irisin in diseases.

2.1. Role of irisin in inducing white fat browning

The upregulation of FNDC5/irisin under aerobic exercise (5, 8, 13) or cold-induced shivering (14) induces the "browning" of white fat *via* increasing the expression of thermogenic genes such as *Prdm16*, *Dio2*, *cidea*, *Cox-7a*, *PGC-1α*, and *UCP-1* in white fat. The activated beige fat dissipated energy in the form of heat by absorbing the excessive energy substrates (fatty acid or glucose), which improved obesity and type 2 diabetes mellitus (T2DM). On the basis of the single-cell RNA-seq method, Kajimura and his workmates revealed that irisin induced adipocyte progenitor cells (APCs) toward *de novo* beige fat biogenesis and proliferation by activating a complex of CD81 and αVβ1/β5 integrins to phosphorylate Focal adhesion kinase 1 (FAK, Tyr397) signaling (15, 16). Moreover, injection of recombinant irisin (r-irisin; 0.5 mg/g/day) into diet-induced obese (DIO) mice for 14 days resulted in activating thermogenic genes expression and browning of subcutaneous white fat, thereby reducing body weight and improving glucose metabolism (3). *In vitro* studies suggested that irisin induced the expression of *UCP1* and other thermogenic genes in primary inguinal adipocytes and 3T3-L1 preadipocytes through activating p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal–related kinase 1/2 (ERK1/2) signaling pathways (3). Inhibition of P38 and ERK1/2 expression could block the upregulation of Uncoupling protein 1 (UCP-1) by irisin, which was called "Irisin ERKs the Fat" by Wu et al. (17). In conclusion, irisin can promote the proliferation and differentiation of beige APCs and the browning of mature white fat.

Most studies have shown that irisin expression is significantly lower in individuals with DIO or T2DM than normal individuals (6, 18–20). However, there are also opposite results, for instance, the concentration of irisin in the blood of obese individuals is higher than that of thin individuals (normal) (21–23) under sedentary conditions. A meta-analysis with 1,005 obese patients and 1,242 control subjects showed that obese individuals had a higher circulating irisin level (24). In the development of obesity, the high level of irisin was probably derived from the increased in white adipose tissue (WAT) (25), as the expression of FNDC5/irisin was decreased in muscle tissues (26), as well as in brown adipose tissue and other tissues. However, if irisin concentration is indeed elevated in obese individuals, then why is not irisin doing its job of burning and "ERK-ing" the fat? In obese adipose tissues, is it reduced in the expression of its receptor αVβ1/β5 integrins or the sensitivity? This controversial phenomenon was also assumed as "irisin resistance". Clearly, these questions require further exploration in the future.

2.2. Role of irisin in the liver

The liver is the main site of gluconeogenesis and glycogenesis to maintain energy metabolism. Irisin expression was significantly reduced in subjects with steatohepatitis (6, 18, 19) or in mouse models of ischemia-reperfusion (I/R)–induced liver injury (27, 28). Long-term exercise-induced irisin or supplementation of exogenous r-irisin could protect the liver from non-alcoholic fatty liver disease (NAFLD) (6, 7), liver glucose disorder (29, 30), or I/R-induced liver injury (31), which embodied the potential role of irisin in muscle-liver cross-talk (32).

Hong et al. (33) revealed that injection of r-irisin into DIO mice for 2 weeks inhibited hepatic cholesterol synthesis *via* activating Adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK, Thr172) and inhibiting sterol regulatory element-binding transcription factor 2 (SREBP2) expression. Hepatic glucose homeostasis is closely related to hepatic gluconeogenesis and glycogen synthesis. Studies have found that irisin reduces the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase)–mediated gluconeogenesis in the liver, which can be weakened by suppressing AMPK Small interfering RNA (AMPK siRNA), suggesting that irisin inhibits gluconeogenesis through activating

AMPK-PEPCK/G6Pase pathway (30). Similar results also revealed that irisin inhibited glucosamine (GlcN) or palmitate-induced primary hepatocyte insulin resistance by activating phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt)/forkhead box protein O1 (FOXO1)–mediated PEPCK and G6Pase; meanwhile, irisin augments liver glycogenesis through PI3K/Akt/GSK3–glycogen synthase (GS) signaling pathway (29). A recent study has revealed that exercise-induced irisin competitively inhibits the binding of myeloid differentiation factor 2 (MD2) and Toll-like receptor 4 by forming a complex with MD2 in liver cells and thus inhibits the inflammatory response, which may contribute to the improvement of NAFLD by reducing liver steatosis and fibrosis through exercise (34).

I/R is a leading reason of liver injury after liver resection or transplantation (35), which highly associated with liver steatosis (36). During I/R, irisin expression was significantly reduced in serum and liver tissues (27, 31). Intravenous injection of irisin (250 μg/kg) significantly attenuated the I/R-induced decrease of mitochondria, the increment of apoptotic liver cells, high expression of inflammatory factors, and oxidative stress in the liver (27). Studies have found that supplemental irisin can bind to integrin αVβ5 and activate the downstream AMPK-UCP2 pathway to protect intestinal epithelial cells against I/R-induced cell apoptosis and oxidative stress (37).

In conclusion, irisin acts as an anti-obesity and anti-diabetic factor *via* regulating glucose and cholesterol synthesis metabolism in the liver. It enhances liver glycogen synthesis by activating PI3K/Akt/GSK3-GS pathway and inhibits gluconeogenesis *via* activating AMPK-PEPCK/G6Pase as well as PI3K/Akt/FoxO1-PEPCK and G6Pase pathway in the liver. In addition, irisin alleviates inflammation and oxidative stress in liver injury induced by I/R. However, there are still some problems that are not clear enough. The role of irisin receptor in the liver is rarely reported, and whether irisin first activates receptors on the surface of hepatocyte and then regulates glucose and lipid metabolism is still unclear.

2.3. Role of irisin in nerves

FNDC5/irisin as a novel therapeutic factor capable of improving cognition, learning, and memory function (38), which has been proved in brain injury caused in cerebral ischemia (39), stroke (40), and anxiety (41). The mediator role of FNDC5/irisin in the brain was first described by Spiegelman and his co-workers in 2013 (8); it was shown that RNA interference (RNAi)-mediated knockdown of FNDC5 reduced brain-derived neurotrophic factor [BDNF; key factor for neuronal cell survival, synaptic plasticity, dendritic arborization, and synaptogenesis (42, 43)]; reversely, increasing irisin levels in the blood by delivery FNDC5 with adenovirus increased the expression of BDNF and other neuroprotective factors, which opened a new avenue in skeletal muscle-brain cross-talk.

Moreover, *fndc5* knockout Knock out (KO) mice had abnormal morphology and function of dentate gyrus neurons (part of the hippocampus), and the cognitive function of the mutant mice was significantly inhibited. Direct or peripheral delivery of irisin to the dentate gyrus was sufficient to ameliorate the cognitive deficits and neuropathology of the mice (44). In the mouse cerebral ischemia model induced by middle cerebral artery occlusion, the level of irisin was negatively correlated with the cerebral infarction volume and brain function injury score, whereas in those treated with r-irisin, the cerebral infarction volume, nerve function injury, and brain edema of the mice were significantly improved, which were related with phosphorylation of ERK1/2-Akt–mediated inflammation (39). Recently, integrin αV/β5 in the hippocampus and cortex was detected (45), which makes it available that irisin combines with integrin αV/β5 to exert the protective effect on brain; however, the specific mechanism remains to be further explored.

In addition, Lourenco et al. showed that the FNDC5/irisin expression was reduced in hippocampi and cerebrospinal fluid of Alzheimer's disease (AD) models. Inhibition of FNDC5 in the brain impaired the memory system of mice and blocked the neuroprotective effect of physical exercise on synaptic plasticity and memory. Conversely, the increased irisin levels in AD mice improved synaptic plasticity and memory (38). *In vitro*, r-irisin prevented neurons from Aβ- (25–35)–induced cell toxicity *via* attenuating IL-6– and IL-1β–mediated inflammation status (46). Furthermore, irisin was the upstream regulator of BDNF, which attenuated the learning and memory deficits as well as the cytotoxic response against Aβ toxicity in AD (47, 48). Thus, the activation of the irisin–BDNF axis may be a potential therapeutic target for AD (49).

In conclusion, the expression of irisin was decreased in patients with brain injury. Exogenous r-irisin supplementation significantly protects nerves and enhances memory and cognitive function. Thus, irisin can be used as a potential target for the treatment of stroke, cerebral ischemia, AD, and other brain injuries.

2.4. Role of irisin in bone

2.4.1. Effects of irisin on bone tissues

Musculoskeletal interaction is one of research hot spots in recent years (50, 51). Colaianni et al. (52) found that conditioned medium (CM) from primary myoblasts of mice after 3 weeks of exercise induced a higher degree of osteoblast differentiation *in vitro* than that under resting conditions, and adding neutralizing antibody of FNDC5/irisin into the CM significantly reduced the expression of alkaline phosphatase (ALP) and collagen I (Col I) in osteoblasts. That was the first to establish that irisin secreted from muscles has a positive regulatory effect on bone during physical exercise. After that, Zhang et al. (53) found that 2 weeks of free wheel-running exercise increased the expression of

osteogenic markers such as osterix (Osx), bone sialoprotein (BSP), and osteocalcin (OCN), as well as FNDC5/irisin in bone tissues. Irisin receptor integrin αV mediated the modulation of irisin on bone during exercise. Eight weeks of running exercise–inhibited ovariectomized (OVX) induced the reduction of femoral trabecular and cortical bone mineral density (BMD) (54), and exercise improved bone microarchitecture and increased the number of ALP-positive cells in OVX mice, whereas twice-weekly injection of cyclo RGDyk polypeptide drugs (anti-irisin receptor integrin αV agents) weakened the improvement effects of exercise (55).

The concentration of FNDC5/irisin was strongly correlated with BMD and bone homeostasis. A cross-sectional and case-control study showed that low concentrations of irisin in serum were related to hip fractures and osteoporosis in postmenopausal women (56, 57). FNDC5/irisin deletion in osteoblast lineage resulted in a lower bone density and delayed bone development and mineralization in mice, FNDC5/irisin KO also blocked the increment of cortical bone thickness by 4 days of voluntary wheel-running exercise (58). Systemic FNDC5 KO mice resulted in low bone strength and mass than Wild type (WT) mice (59), and global FNDC5/irisin KO also completely blocked OVX-induced osteocytic osteolysis and trabecular bone loss (60).

2.4.2 Role of irisin in osteoblasts

Bone modeling and remodeling require the balance of osteoblasts-induced bone formation and osteoclasts-induced bone resorption (61). Irisin activates osteogenic gene expression and induces bone formation. Injecting r-irisin (100 μg/kg/weeks, 4 weeks) significantly increased the mRNA expression of activating transcription factor 4 (*Atf4*) in bone marrow and phosphoprotein 1 (osteopontin, Spp1) in the whole tibia, indicating that irisin shifted from mesenchymal stem cell commitment toward osteoblast lineage and increased bone formation; *in vitro* experiments showed that r-irisin upregulated osteoblast marker genes like *Bmp2/4*, *Spp1*, Runt-related transcription factor-2 (*Runx2*), *Alp*, and *Atf4*, as well as phosphorylation of ERK1/2 in bone marrow stromal cells (62).

Furthermore, administration of r-irisin (100 ng/ml) induced differentiation and mineralization of primary rat osteoblasts and MC3T3-E1 cells by increasing the expression of osteoblast transcription regulators and differentiation marker, which was blocked by inhibiting p38 and ERK1/2 expression (63). Physical exercise activated Akt-β-catenin (essential for osteoblastic differentiation (64)) and induced ALP-positive cells increment, and these effects were abolished by tail vein injecting integrin αV inhibitor (55), which suggested that irisin increased bone mass by binding to osteoblast surface receptors and activating the Akt/β-catenin-Alp pathway. Recently, Xue et al. (65). also got a similar result in preosteoblasts.

2.4.3. Role of irisin in osteoclasts

Irisin protects bone microstructure by stimulating osteoblasts production and inhibiting the differentiation of osteoclasts to establish a "new balance". Injection of r-irisin into OVX-induced mice significantly increased the number of osteoblasts on the surface of trabeculae bone while inhibiting the number of osteoclasts and decreasing the concentration of tartrate-resistant acid phosphatase (TRAP; marker of osteoclasts) (66, 67).

In addition, supplementation of r-irisin (20 nmol/L) in pre-osteoclastic RAW264.7 cells for 4 days resulted in the decrease of osteoclast differentiation markers (53). Moreover, Ma et al. (68) showed that irisin promoted the proliferation of two osteoclast precursor cells (RAW264.7 cells and mouse bone marrow monocytes) *via* activating p38-MAPK and c-Jun N-terminal kinase (JNK) signaling pathways but significantly downregulated osteoclasts differentiation markers, as well as decreased hydroxyapatite resorption pits and $TRAP^+$ multinucleated cell numbers. However, there were also some different results. Estell et al. (69). found that administration of irisin (2–10 ng/ml) promoted the differentiation of mouse bone marrow progenitors toward osteoclasts and that overexpression of fndc5 in mice promoted the differentiation and resorption of osteoclasts, which resulted in lower bone mass.

2.4.4. Role of irisin in osteocytes

Osteocytes accounted for more than 90% of bone cells and played crucial roles in bone homeostasis. Irisin prevents bone loss and osteoporosis by robustly inhibiting osteocytic apoptosis. Spiegelman and his workmates revealed for the first time that irisin bound directly to osteocytes by integrin receptors (αVβ1/β5) and that inhibition of integrin αV receptor expression significantly inhibited the activation of SOST in bone cells by r-irisin. Injection of r-irisin (100 μg/kg) *in vivo* improved disuse-induced low viability and apoptosis of osteocytes and a high rate of empty lacunae (70). Furthermore, they found that irisin rapidly activated the expression of *Atf4* and inhibited apoptosis by activating ERK1/2 in MLO-Y4 osteocytes, which contributed to bone development (70, 71).

In summary, irisin regulates bone regeneration and homeostasis, which reflects the key regulatory role of muscle on bone (72). We summarized the effects of irisin in bone tissue cells in Table 1.

2.5. Role of irisin in skeletal muscle

AMPK–PGC-1α (PPARγ coactivator-1α)–FNDC5 axis is the most important pathway for irisin synthesis. During exercise, the Ca^{2+} level is increased significantly in the muscle cytoplasm along with skeletal muscle contraction and then stimulates the phosphorylation of AMPK (78), which, in turn,

TABLE 1 Role of irisin in bone.

Type cell/Animal	Irisin concentration/Endurance	Main effect	Reference
Mice	100 μg/kg/week; 4 weeks	Atf4↑, spp1↑, bone formation↑	(62)
Primary osteoblast and MC3T3-E1 cell	100 ng/ml; 3 and 14 days	Runx2↑, Osx↑, ALP↑, ColIa1↑, p-P38↑, p-ERK1/2↑	(63)
Murine BMSCs	40 μM; 2, 7, 14, and 21days	Runx2↑, OCN↑, ALP↑, Atg5↑, β-catenin↑, Lef1↑, Tcf4↑	(73)
Mouse preosteoblast-like cells MC3T3-E1	100n g/ml; 1, 5, 10, and 20 min; 3, 8, and 24 h; 6 days	P21↓	(74)
Mice	100 μg/kg/week; 4 weeks	ALP↑, Col I↑, BMD↑	(10)
Primary murine OC, MC3T3E1	100 ng/ml; 14 days	Runx2↑, Atf4↑, Osterix↑, Col I↑, Osteoprotegerin↑, Trap (×), Cathepsin K (×)	(75)
Primary osteoblasts, MC3T3-E1	1 nM; 24, 48, and 72 h; 14 days	ColIa1↑, ALP↑, calcium deposition↑, β-catenin↑,	(76)
RAW264.7 cells	20 nmol/L; 4 days	NFATc1↓, CK ↓, Trap↓	(53)
RAW264.7 cells, mouse bone marrow monocytes	20 and 40 nM; 4 and 5 days	RANK↓, CK ↓, Trap↓ differentiation↓	(68)
MLO-Y4	100 ng/ml; 1, 5, 10, 20, and 60 min; 6 days	p-ERK1/2↑, Atf4↑, SOST↓, caspase3/9↓	(70)
Mice	18 ng/ml; 3× a week for 4 weeks	TNF-α↓, IL-17↓	(77)

↑: Increased, ↓: Decreased, ×: No change

enhances PGC-1α and regulates transcription of downstream factors such as *fndc5*. Shan et al. revealed that myostatin (*MSTN*) KO in skeletal muscle significantly increased PGC-1α and FNDC5/irisin expression, and the high level of irisin increased the browning of WAT in $MSTN^{-/-}$ mice (79). Moreover, Ge et al. found that myostatin inhibited FNDC5/irisin expression by increasing miR-34a (80).

The blood circulation level of irisin has been identified as a biomarker for muscle mass and performance (81). For example, the concentration of irisin in patients with sarcopenia and pre-sarcopenia was lower compared with that in non-sarcopenic participants (82, 83). Exposure to an ambient hypoxic environment can cause skeletal muscle loss and atrophy, along with the low concentration of irisin in blood circulation both in humans (84) and mice (85), which could be one of the reasons of muscle atrophy induced by hypoxia (86). However, interestingly, knockdown of fndc5 in skeletal muscle still performed equal muscle mass, development, growth, regeneration, and strength compared with WT mice. Although, there was no difference in cardiotoxin-induced muscle injury between fndc5-mutant and WT mice (87).

Multiple studies showed that exogenous r-irisin improved skeletal muscle loss and atrophy. Colaianni et al. revealed that r-irisin prevented hindlimb unloading-induced muscle mass decline and decrease of myosin type II expression (10). In addition, *in vitro*, *fndc5* gene expression and irisin concentration were positively correlated with the process of differentiation of C2C12 myotubes; r-irisin supplementation increased human primary skeletal muscle cell growth and hypertrophy by increasing insulin-like growth factor 1(IGF-1)/PGC1α4 and decreasing myostatin through activating ERK1/2 pathway (88). Another study from Reza et al. showed that r-irisin increased myogenic differentiation and myoblast fusion *via* activating IL-6 signaling pathway, and r-irisin treatment also improved denervation-induced muscle injury by increasing protein synthesis through the ERK1/2 pathway (9). Irisin treatment (100 ng/ml, 24 h) also prevented dexamethasone-induced atrophy in C2C12 myotubes by upregulating IGF-1 and attenuating proteolytic activity through dephosphorylation of FoxO3α-mediated ubiquitin-proteasome overactivity (89).

In short, irisin is mainly produced by muscle tissue *via* Ca^{2+}–AMPK–PGC-1α–FNDC5 pathway. It induces the expression of myoblasts by activating downstream ERK1/2 and IL-6 pathways in an autocrine manner, which plays key regulatory role in muscle growth and differentiation. However, there are still some problems, such as whether the receptor is still integrin αV/β5 on the surface of muscle cells. In addition, the role of integrin αV/β5 in exercise-induced muscle hyperplasia and hypertrophy is unclear.

2.6. Role of irisin in articular cartilage

Osteoarthritis (OA) is a degenerative joint injury characterized by joint pain, progressive cartilaginous degeneration, and stiffness, which poses a great challenge to the physical health of the patients (90). It was stated that FNDC5/irisin activated by moderate physical exercise played a key role in alleviating symptoms and the process of OA such as progressive cartilaginous degeneration, synovial inflammation, and osteophyte formation (71, 91, 92). Studies have shown that the expression of FNDC5/irisin is reduced in patients with osteoarthritic cartilage (93) or synovial fluid (94) compared with that in healthy subjects. In addition, FNDC5 KO mice accelerated anterior cruciate ligament transection–induced OA progression; conversely, FNDC5 knock-in attenuated OA progression (95).

Direct intraarticular injection of irisin may be more effective as almost no blood vessels pass through cartilage. Destabilized medial meniscus (DMM)–induced OA mice were directly injected intra-articular with r-irisin for 8 weeks; the results showed that irisin prevented articular cartilage loss and ameliorated irregular gait; moreover, administration of irisin increased autophagy flux and survival of chondrocytes in DMM-induced OA mice by increasing the expression of LC3 and proliferating cell nuclear antigen (93). *In vitro*, Col II and tissue inhibitor of matrix metalloproteinase (MMP)–1 and –3 expression was significantly increased, and Col X (hypertrophic chondrocyte–related gene) and MMP-1 and MMP-13 expression significantly decreased by adding r-irisin to human primary chondrocytes for 7 days, indicating that the addition of irisin contributes to maintaining partial stability of the extracellular matrix (ECM) of cartilage (96). This mechanism may be related to irisin, lowering the activation of p38, JNK, and Akt in chondrocytes (96, 97). Recently, Jia et al. (98). found that exercise-activated irisin alleviated OA chondrocyte inflammation by inhibiting PI3K/Akt/Nuclear factor kappa B (NF-κB) signaling pathway and suppressing the NOD-like receptor protein 3 (NLRP3) and caspase-1–mediated pyroptosis. In addition, *in vitro*, r-irisin treatment (5 and 10 ng/ml, 24 h) attenuated IL-lβ–induced PI3K/Akt/NF-κB p65 cascade and blocked the nuclear translocation of NF-κB p65. Furthermore, irisin supplementation also improved the inflammatory status of OA by reducing the expression of inflammation factors such as IL-1β (95, 99), TNF-α (92), IL-6, and IL-1 (96).

In summary, the expression of irisin was reduced in patients with OA, and moderate physical exercise could alleviate OA by activating irisin (92). The therapeutic effect of irisin is mainly reflected in reducing the inflammatory state of damaged cartilage and increasing the autophagy flux; additionally, intraarticular injection of r-irisin may be effective for rehabilitating patients with OA.

2.7. Role of irisin in cancer

Cancer is one of the leading causes of human death. Regular exercise helps reducing the risk of cancer (100); as an exercise gene (101), the role of FNDC5/irisin in the occurrence and prevention of cancer has received extensive attention (102). Most studies have shown an elevated irisin expression in cancer (103–105). However, a few studies also reported that irisin expression is reduced in patients with cancer (106). Therefore, more research studies are needed to explore the role of irisin in cancer.

In vitro, r-irisin inhibited the proliferation, migration, invasion, and epithelial-to-mesenchymal transition (EMT) in lung cancer (11), epithelial ovarian cancer (107), and pancreatic cancer (PC) (108) cells by inhibiting PI3K/Akt- and Signal transducer and activator of transcription 3 (STAT3)-mediated (109) downstream Snail expression (an important role in stimulating EMT).

Irisin induces the arrest of cancer cell division and inhibits cell growth. Huang et al. revealed that irisin induced G (2)/M cell cycle arrest and increased the expression of P21 and tissue factor pathway inhibitor 2, thereby inhibiting the proliferation and invasion of glioblastoma multiforme cells (91). Similarly, Liu et al. found that the receptor of irisin also existed on the surface of PC cells; supplementation of both non-glycosylated and glycosylated r-irisin in PCs could induce G1 arrest and inhibit the growth of PC *via* activating AMPK and inhibiting mTOR expression (110); these results indicate that irisin can affect tumor tissues and exert antitumor properties. However, there is still not enough evidence that irisin can directly act on the integrins on tumor cells to inhibit the development of EMT or tumor proliferation (111).

Overall, irisin has a wide application prospect for the treatment of cancer. Irisin inhibited the proliferation, migration, and invasion of tumor cells by inhibiting PI3K/Akt- and STAT3-mediated Snail/EMT pathways. In addition, irisin also inhibited tumor growth by inducing G1 or G (2)/M cell cycle arrest through AMPK/mTOR pathway.

2.8. Role of irisin in myocardium and blood vessel

CVDs include hypertension, coronary artery disease, myocardial infarction, heart failure, atherosclerosis, and myocardial I/R injury, which are the leading cause of human death worldwide (112). Regular exercise can reduce the risk of CVDs, and irisin may play a crucial role in it. Studies have found that the expression of irisin in patients with CVDs is significantly lower than that in healthy people (113–117). Li et al. revealed that resistance exercise could activate the release of irisin from skeletal muscle and then stimulate the AMPK-PINK1/Parkin-LC3/P62 signaling pathway, which regulated mitophagy and inhibited oxidative stress in the myocardium (12). *In vitro*, studies have shown that irisin binds directly to the endothelial cell surface receptor integrin αV/β5, thereby phosphorylating AMPK (Thr172) and activating PGC-1α (induce mitochondrial biogenesis) and mitochondrial transcription factor A (a key activator of mitochondrial transcription and a participant in mitochondrial genome replication).

Cardiac hypertrophy progresses to heart failure; irisin can significantly improve myocardial hypertrophy. Qing et al. showed that administration of r-irisin could attenuate angiotensin II (Ang II)–induced cardiomyocyte hypertrophy, *in vitro*, and that treatment of irisin in transverse aortic constriction (TAC)–induced cardiac hypertrophy murine, *in vivo*, significantly suppressed cardiac hypertrophy and fibrosis by phosphorylating AMPK (Thr172) and inhibiting the

phosphorylation of mTOR (Ser2448). However, the expression of irisin increased in the hypertrophic heart and serum during this period, which may be a stress response from the body, as the elevated irisin could decrease endothelial damage by suppressing oxidative stress and inflammation (4, 118). Yue et al. found that r-irisin protected myocardial hypertrophic mice induced by TAC or Ang II–treated cardiomyocytes *via* inhibiting NLRP3-mediated pyroptosis (119).

The therapeutic role of irisin on cardiac hypertrophy was also reflected in the improvement of autophagy flux and induction of protective autophagy. Li et al. found that supplementation of irisin in Ang II–treated cardiomyocytes significantly increased the expression of LC3II and decreased P62 expression and activated the phosphorylation of AMPK (Thr172) and ULK1 (Ser555), thereby reducing cardiomyocyte apoptosis, and this protection will be reversed by autophagy inhibitor such as 3-methyladenine, autophagy-related 5 siRNA (ATG5), and chloroquine; moreover, blockage of AMPK and ULK1 also abrogated autophagy flux and indicted irisin-induced protective autophagy in cardiac hypertrophy *via* activating AMPK-ULK1 pathway (120, 121).

Growing evidence suggests that the content of irisin in patients with atherosclerosis is significantly lower than that in normal controls (122–124), and irisin supplementation has a significant effect on the treatment and improvement of atherosclerosis. For example, irisin supplementation can significantly improve endothelial dysfunction, decrease endothelial apoptosis, and predominantly decrease atherosclerotic plaque area in nicotine or streptozotocin-induced apolipoprotein E-Null [apoE($^{-/-}$)] atherosclerosis mice (125). Here, we enumerated the role of irisin in atherosclerosis disease in Table 2.

Overall, the integrin αVβ5 on the endothelial cell surface could be activated by FNDC5/irisin. As a key energy sensor to maintain energy balance and mitochondrial hemostasis (131), AMPK mediated the effect of FNDC5/irisin on mitophagy, oxidative stress, and mitochondrial biogenesis, thereby improving myocardial hypertrophy, myocardial infarction, atherosclerosis, and other cardiac diseases, which reflecting the protection of regular exercise on cardiac health.

2 Conclusions

In this review, we systematically summarized the roles of FNDC5/irisin in fat, liver, nerve, bone, skeletal muscle, articular cartilage, cancer, and angiocarpy. Irisin, as a muscle factor secreted by exercise, plays an extremely important role in regulating fat browning, improving liver and systemic glucose metabolism, maintaining musculoskeletal homeostasis, promoting synaptic growth, and inhibiting the progression of cancer. The mechanism of irisin is mainly through first directly binding to its receptor integrin αV/β1/5 and then activating AMPK, FAK, and MAPK signaling pathways. Collectively, potential mechanisms and signaling pathways for the actions of irisin in musculoskeletal and pathological tissues are shown in Figures 1 and 2, respectively.

There are still some unsolved questions, for example, the concentration of irisin in pathophysiological conditions that are highly controversial; some studies suggest that the irisin level rises in patients with obesity or cancer, but why does irisin not play a role in burning and "ERK-ing" the fat as well as inhibiting the development of cancer? Perhaps, its receptor sensitivity and number are reduced under these pathological conditions, which resulted in "irisin resistance". At this point, high concentration of irisin may not come from muscle tissue but from newly increased fat or cancer tissues; perhaps, due to the decreased activity and expression of its receptor, irisin could not play a substantial role even if the concentration increased. In addition, whether irisin that directly binds to receptors on the surface of chondrocytes, myoblasts, cancer cells, and hepatocytes plays a regulatory role is still unclear, and relevant studies are limited. Therefore, it may be necessary to further explore the role of irisin by detecting the expression of its receptor integrin αV/β1/5 in these pathological and physiological tissues.

Here, we summarized the progress and mechanism of FNDC5/irisin in physiological and pathological conditions,

TABLE 2 The role of irisin in atherosclerosis.

Type cell/Animal	Irisin concentration/ Endurance	Main effect	Reference
APOE$^{-/-}$ mice	0.02 μg/μl, 2× a week for 3 weeks	Irisin reversed intimal thickening *via* integrin αVβ5 receptor.	(125)
		Irisin inhibited atherosclerosis progression *via* the integrin αVβ5/PI3K/P27 pathway.	(126)
C57BL/6, human umbilical vein endothelial cells	20 nM for 7 days in mice, 24 h in EC	Irisin increased EC viability, migration, and tube formation *via* Akt/mTOR/ Nrf2 pathway.	(127)
ApoE$^{-/-}$ mice	0.02 μg/μl, 2× a week for 4 weeks	Irisin decreased endothelial apoptosis, and predominantly decreased atherosclerotic plaque area.	(128)
RAW264.7 macrophages	20, 40, and 80 ng/ml for 30 min	Irisin reduced lipid accumulation in macrophages and inhibited apoptosis	(129)
Human umbilical vein endothelial cells	0.01, 0.1, and 1 μg/ml for 48 h	Irisin ameliorated inflammation and endothelial dysfunction by inhibiting ROS-NLRP3.	(130)

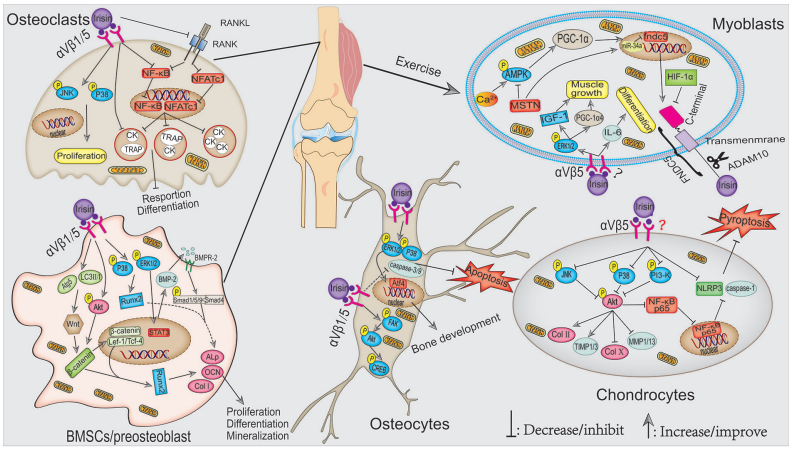

FIGURE 1
Potential mechanisms signaling pathways for the actions of irisin in musculoskeletal. During exercise, the elevated Ca^{2+} in muscle cytoplasm-induced activation of the AMPK–PGC-1α–FNDC5 axis is the main pathway for irisin synthesis. In addition, irisin, in turn, can stimulate muscle growth and myoblast differentiation *via* ERK1/2–IGF-1/MSTN and IL-6 signaling pathways, respectively. Multiple pathways mediated exercise-induced irisin and r-irisin–activated osteoblast differentiation and mineralization, e.g., p38/ERK1/2, Akt-β-catenin, and Wnt-β-catenin–mediated activation of ALP/OCN/Col I pathways. In osteoclast, irisin induced its proliferation through activating the p38/JNK pathway. In addition, irisin also inhibited the NF-κB and NFATc1 levels in the nucleus, thus inhibiting the expression of osteoclast differentiation marker genes. As for osteocytes, irisin inhibited osteocyte apoptosis by inhibiting caspase-9 and caspase-3 expression, which probably through activating p38/ERK1/2. Furthermore, moderate exercise-activated irisin or r-irisin could alleviate OA by maintaining ECM stabilization and reducing inflammatory response through p38/JNK-Akt and PI3K/Akt/NF-κB signaling pathway, respectively.

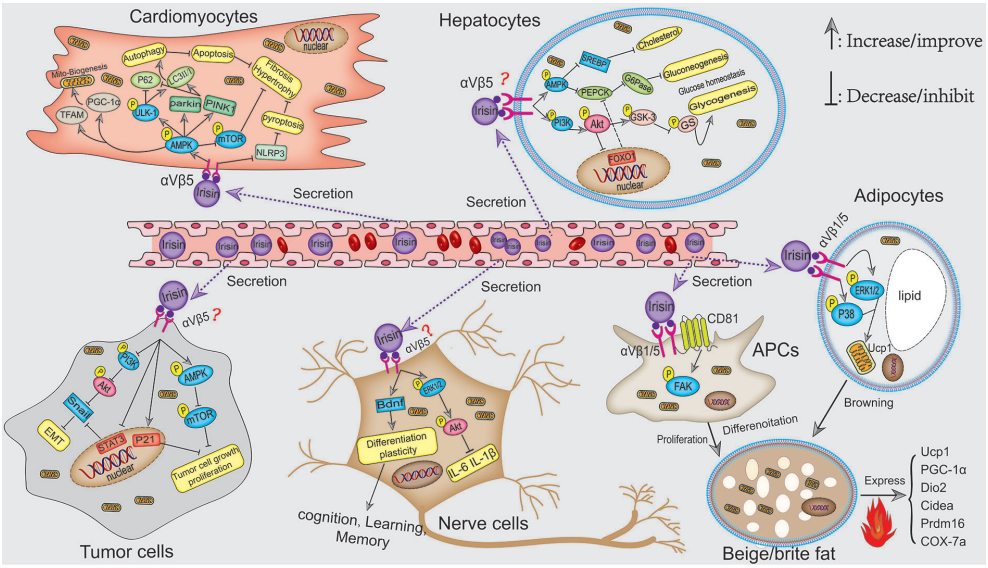

FIGURE 2
Potential mechanisms signaling pathways for the actions of irisin in the pathological tissues. Irisin protects against DIO by inducing the recruitment of beige fat to dissipate energy into heat. This mechanism is involved in p38 MAPK and ERK1/2 pathways, as well as FAK-mediated beige APCs proliferation. In addition, irisin attenuated diet-induced metabolic disorders, including NAFLD and hepatic steatosis by promoting the synthesis of liver glycogen *via* PI3K/Akt/GSK3-GS and inhibiting the generation of liver gluconeogenesis through AMPK-PEPCK/G6Pase and PI3K/Akt/FOXO1-mediated PEPCK/G6Pase pathways. In brain tissues, irisin promoted cognition and neuro development *via* inhibiting the inflammatory response and activating BDNF-mediated nerve cell survival, differentiation, and plasticity. Moreover, irisin affects the proliferation, migration, and invasion of tumor cells probably by binding integrin αV/β5–mediated PI3K/Akt-Snail-EMT and AMPK-mTOR pathways, which has great therapeutic prospects for inhibiting cancer development. Moreover, exercise-induced irisin can also reduce the risk of cardiovascular diseases. In cardiomyocytes, irisin stimulated AMPK-mediated autophagy and mitobiogenesis by binding to its receptor integrin αV/β5, thereby relieving cardiac hypertrophy and injury.

and we analyzed the shortcomings of current research of FNDC5/irisin. We hope that this review may provide an available reference for FNDC5/irisin research.

Author contributions

SL and FC were mainly involved in data analysis and manuscript drafting, KN and DW helped to draw the mechanic images. ZW and PF made final checks on the manuscript and data. HX provided major theoretical knowledge and relevant suggestions. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

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