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Stars and rising stars in pediatric endocrinology 2022

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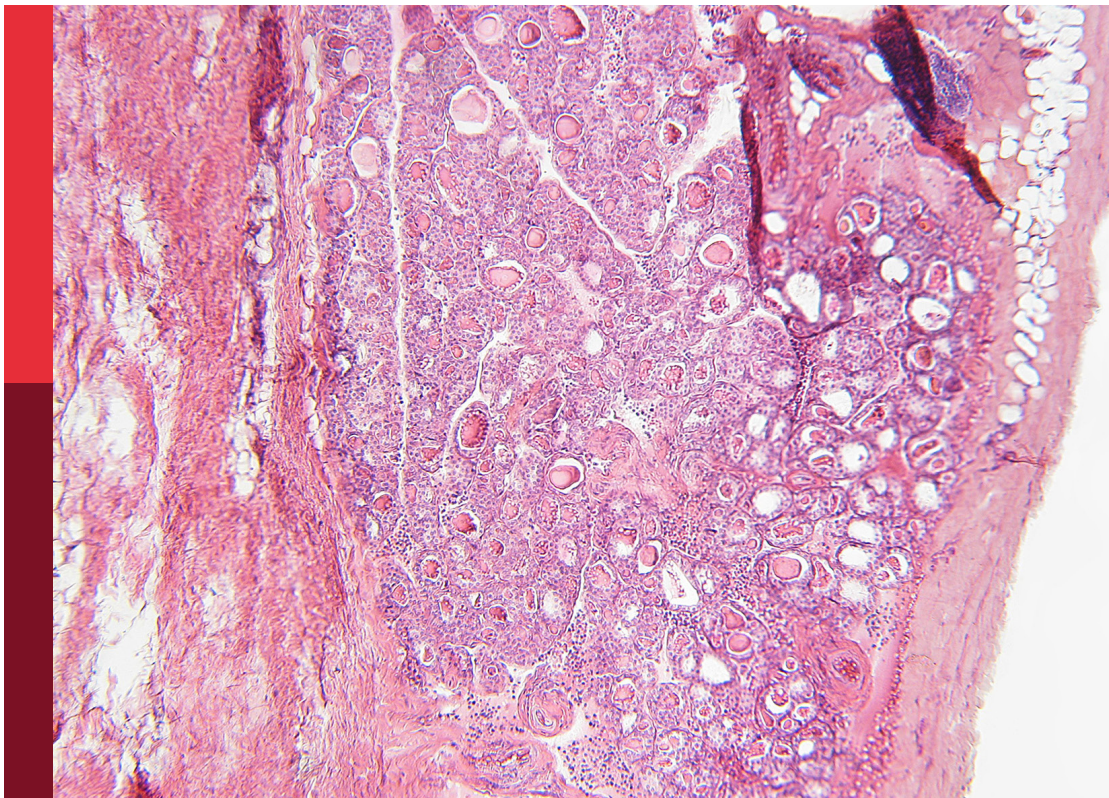

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Stars and rising stars in pediatric endocrinology: 2022

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Editorial: Stars and rising stars in pediatric endocrinology: 2022

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continuous glucose monitor, wolfram (DIDMOAD) syndrome, disorders of sex development (DSD), COVID - 19, hyperlipidemia, type 1 diabetes, honeymoon phase, multiple endocrine neoplasia

Editorial on the Research Topic
Stars and rising stars in pediatric endocrinology: 2022

Endocrinology is an evolving field, and this Research Topic, *Stars and Rising Stars in Endocrinology*, was offered to attract the works of the best brains in the field. The Rising Stars are mid-level to upper-level clinicians and scientists who are making their mark in Endocrinology. This *Editorial* covers research work from diverse areas of endocrinology that range from the genetics of rare endocrine disorders to novel applications of diabetes technology and a new theory of dyslipidemia in endocrinology. Each of these works is forward-leaning and promises a wider application in endocrinology. From the newly proposed theory of hyperlipidemic memory of type 1 diabetes to the use of whole-exome sequencing to identify a novel mutation causing a disorder of sexual development, and the use of transcriptomic data to diagnose growth hormone (GH) deficiency in children, this Research Topic contains innovative works that will shape the future of endocrinology.

Several articles focused on improving the diagnostic approaches to endocrine disorders. Garner et al. report on innovative techniques to improve the diagnosis of GH deficiency (GHD) in children with short stature. Currently, the diagnosis of GHD in children is imprecise as the gold standard test called the GH stimulation test is non-physiologic and has many shortcomings (1). They describe how an accurate diagnosis of GHD can be made using gene expression signatures in peripheral blood in a transcriptomic modality that combines gene expression data and random forest analysis. This new technique promises to increase the accuracy of diagnosing GHD and could potentially replace pharmacologic stimulation tests. Frontino et al. seek to expand the diagnostic criteria for Wolfram syndrome (WS) to include hypergonadotropic hypogonadism. WS is a rare autosomal recessive disease that presents classically with non-autoimmune diabetes mellitus, optic atrophy, diabetes insipidus, and deafness (2). These investigators report a higher prevalence of primary hypogonadism in children with WS and suggest that the increased occurrence of hypogonadism in WS warrants its inclusion in the diagnostic criteria to enable earlier detection and diagnosis. Wan et al. propose that an accurate diagnosis of disorders in sex differentiation (DSD) requires the detection of candidate genes as the genetic etiology of most individuals with DSD is unclear (3). These investigators report patients with DSDs whose whole-exome sequences identified a novel mutation that might signal via the

upregulation of the β-catenin protein. These new diagnostic techniques will strengthen and possibly replace existing modalities.

The next set of articles focused on expanding the understanding of alterations in classic disease phenotypes through avenues such as the impact of COVID-19 on sexual maturation, emerging phenotypes in the diagnosis of insulinoma, reassuring findings on palpable breast tissue in infants, and the exciting impact of continuous glucose monitors (CGM) in non-diabetic conditions. Chioma et al. build on the hypothesis that the COVID-19 pandemic triggers pathological processes in humans (4, 5) to report a higher prevalence of rapidly progressive central precocious puberty (CPP) in girls during the COVID-19 pandemic from 2019 to 2022. Their finding that the prevalence declined after the peak of the pandemic suggests that the SARS-CoV-2 virus, or pandemic-associated environmental and psychosomatic changes could play a role in triggering CPP in girls. Melikyan et al., in another disease clarifying article, report an increased occurrence of multiple endocrine neoplasia type 1 (MEN 1) syndrome and Grade 2 tumors in children with insulinomas. The authors found that children with MEN 1 had a significantly higher number of pancreatic tumors when compared to those with sporadic insulinoma. Furthermore, family members of patients with MEN 1 had increased MEN 1 manifestations such as neoplasia of the parathyroid glands. They conclude that all children diagnosed with insulinoma should receive genetic testing, along with their family members, to exclude malignancies. They further recommend long-term follow-up of these patients. Another disease clarifying article from the Copenhagen Minipuberty Study explains that palpable breast tissue in infancy may be a benign process that does not require endocrinological investigation. This reassuring finding should prevent unnecessary evaluation in these infants and could lead to significant health care savings as the differential diagnosis of palpable breast tissue in infants ranges from benign physiological processes to severe pathologies such as hormone-secreting tumors (6). A close longitudinal follow-up will distinguish benign processes from other more serious conditions such as central precocious puberty and neoplasms. Buchanan et al. describe the first patient with an advanced bone age likely caused by elevated 11-ketotestosterone levels in Wiedemann-Steiner syndrome (WSS). This is important as the mechanism of advanced skeletal maturation is unclear in WSS (7). Sivasubramanian et al. examine the use of CGM in hyperinsulinemic hypoglycemia given that the use of CGM has revolutionized diabetes care and has enabled patients to monitor their glycemia in real time (8). They reported the feasibility and accuracy of CGM in children with hyperinsulinemic hypoglycemia. The safety and efficacy of this intervention in a non-diabetes-related field opens the field for the use of CGM for expanded indications such as in cystic fibrosis, glycogen storage diseases, and neonatal diabetes mellitus.

Nwosu's new theory of hyperlipidemic memory of type 1 diabetes (T1D) is based on 4 clinical studies conducted in children, adolescents, and adults during their partial clinical remission (PR) phase of T1D. This theory explains the dichotomy in atherosclerotic cardiovascular disease risk between subjects with T1D who experienced PR, (remitters), and those who did not, (non-remitters) (9). This theory, which explains the lipid-based *macrovascular* complications of diabetes, complements the earlier theory of hyperglycemic memory which explains the glucose-based *microvascular* complications of T1D between the remitters and non-remitters. Nwosu's hyperlipidemic memory theory and his concept of PR imprimatur have opened the field to inquiry into dyslipidemia in T1D based on PR history. It is conceivable that these inquiries could lead to changes in the guidelines for the prevention and management of dyslipidemia in T1D based on the observed dichotomy in lipid profile between the remitters and non-remitters.

In conclusion, the articles in this Research Topic will have lasting impact on the future of endocrinology. We thank all the Stars and Rising Stars in Endocrinology for their contributions.

Author contributions

BN: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Conceptualization.

Conflict of interest

The author declares 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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HYPOTHESIS AND THEORY
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The Theory of Hyperlipidemic Memory of Type 1 Diabetes

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Literature Search Criteria: A literature search was conducted to identify publications addressing the early phases of lipid phenotypes in children and adults with either type 1 diabetes or type 2 diabetes. Medline, EMBASE, and Ovid were searched using the following search terms: *clinical remission, partial remission, partial clinical remission, honeymoon phase, C-peptide, type 1 or 2 diabetes, children, pediatric type 1 or 2 diabetes, and paediatrics type 1 or 2 diabetes, adults, adult type 1 or type 2 diabetes.*

Partial clinical remission (PR) of type 1 diabetes (T1D) is characterized by continued endogenous production of insulin and C-peptide following the diagnosis and the introduction of exogenous insulin therapy. PR is associated with improved glycemic control and reduced prevalence of diabetes complications. The theory of hyperglycemic memory was proposed to explain this concept of improved glycemic outcomes in remitters (those who experienced PR) versus non-remitters (those who did not experience PR). However, this theory is incomplete as it does not explain the dichotomy in early lipid phenotypes in T1D based on PR status, which is an understudied area in diabetology and lipidology. To fill this knowledge gap, we propose the Theory of Hyperlipidemic Memory of T1D. This theory is premised on our 5-year research on early post-diagnostic dichotomy in lipid phenotypes between remitters and non-remitters across the lifespan. It provides a more rigorous explanation for the differences in lifelong atherosclerotic cardiovascular disease (ASCVD) risk between remitters and non-remitters. We conducted 4 clinical studies in pediatric and adult subjects with diabetes mellitus to characterize the particulars of the hyperlipidemic memory. In the first investigation, we explored the impact of the presence or absence of PR on lipid parameters in children and adolescents with T1D. In the second, we investigated whether pubertal maturation influenced our findings in T1D; and whether these findings could be replicated in healthy, non-diabetic children and adolescents. In the third, we leveraged our findings from T1D and controls to investigate the mechanisms of early lipid changes in T2D by comparing the earliest lipid phenotype of subjects with type 2 diabetes (T2D) to those of remitters, non-remitters, and controls. In the fourth, we investigated the impact of PR on the earliest lipid phenotypes in adults with T1D and compared these early lipid data to those of T2D subjects and controls. This body of work

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across the lifespan in children, adolescents, and adults supports the Theory of Hyperlipidemic Memory. This new theory clarifies why PR largely determines the risks for early-phase dyslipidemia, mid-term microvascular disease risk, and long-term ASCVD risk in subjects with T1D.

Keywords: type 1 diabetes, type 2 diabetes, adults, honeymoon phase, partial clinical remission, cardiovascular disease risk, dyslipidemia, hyperlipidemia

INTRODUCTION

Diabetes mellitus affects 34.2 million Americans, or 10.5% of the population (1). Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of death in individuals with diabetes. In 2017, the morbidity and mortality from ASCVD resulted in an estimated $37.3 billion in healthcare costs in diabetes associated expenditures (1). More than 50% of patients with type 2 diabetes (T2D) have pre-existing CVD at the time of diagnosis (2). But these early CVD prevalence data are unclear in patients with type 1 diabetes (T1D) (3), where mortality from coronary artery disease is approximately 3- to 10- fold higher than in the general population (2).

Despite the strong correlation between ASCVD and diabetes mellitus, the underlying mechanisms remain poorly understood (4), especially in T1D where 50% of the subjects undergo partial clinical remission (PR) or honeymoon phase following the diagnosis. However, the impact of PR on the earliest lipid phenotypes in adults with diabetes mellitus is not known (5). Though PR has been reported to modulate the degree of early-phase dyslipidemia (6), mid-term microvascular disease risk (7), and long-term ASCVD risk (8), no prior study in adults (5) has directly compared the earliest phenotype of lipid-based ASCVD risk between subjects with T2D and T1D, after stratifying the T1D subjects into remitters and non-remitters based on their PR history. Such studies are important to establish the prevalence of dyslipidemia in T1D. These studies will also help to characterize some yet unexamined contributors to diabetic dyslipidemia in children and adults with diabetes mellitus, such as the role of hyperlipidemic memory on subsequent lipid phenotypes (**Figure 1**).

A Literature Review of Current Knowledge in PR

The Diabetes Control and Complications Trial reported a protective role for C-peptide on vasculature in remitters or patients with T1D who had residual β-cell function (8). The Medalist study (9) reported that among adult patients with T1D for >50 years, there is a cohort that still produced insulin, and that this cohort had better glycemic control and lipid profile when compared to their peers. The T1D Exchange study (10) of 919 showed that a great proportion of children and adult patients with T1D were still producing insulin. This study found that residual C-peptide remained 3-5 years after diagnosis in 78% of participants who were diagnosed at >18 years and 46% of those diagnosed at <18 years. Additionally, they found that 6% of subjects with childhood onset-, and 16% of those with adult-onset T1D had residual C-peptide at >40 years after their diagnosis. Despite these landmark findings, there are very sparse data on the characterization of early-phase, post-diagnostic lipid phenotypes in remitters and non-remitters (6) across the lifespan in both children and adults to form a foundational basis for extrapolations to the clinical significance of PR with respect to ASCVD. A review of current literature on dyslipidemia in children and adolescents with T1D shows no consensus on their lipid phenotype, and it is believed that a lack of stratification of subjects by PR history may have confounded these results (11–14). Similarly, a review of the literature in adults subjects with diabetes mellitus showed that while the risk factors for ASCVD are well established in T2D (1), they are less clear in those with T1D (3, 4).

The lack of a detailed analysis of the degree of dichotomy in early lipid phenotypes in T1D subtypes: remitters and non-remitters, and the assumption that subjects with T2D have worse lipid profiles than those with T1D have hindered a thorough assessment of the intrinsic disparities in lipid phenotypes in T1D (1, 3, 4).

Poor Characterization of ASCVD Risk in T1D

Risk factors for ASCVD are well established in T2D (1), but not in T1D (3, 4). Current knowledge indicates that HbA1c, diabetic nephropathy, hypertension, and dyslipidemia are important risk factors for ASCVD in adults with established T1D (15). However, the phenotype of the earliest ASCVD risk profile at the time of diagnosis of T1D compared to T2D, and the cardinal role of PR on early lipid phenotype in T1D, which presages later ASCVD risk status, are not fully characterized.

Diabetic dyslipidemia, the major link between diabetes mellitus and ASCVD, occurs in the setting of low HDL-cholesterol (HDL-C), high fasting and/or postprandial triglycerides (TG), average to high LDL-C, and predominantly small dense LDL-C particles (16). Elevated non-HDL-C correlates with 99% increased CVD risk for patients with T2D (17) where the classic lipid abnormalities are characterized by elevated TG, small dense LDL-C, and low HDL-C (18). Similarly, CVD risk in T1D is predicted by total cholesterol/HDL cholesterol, and non-HDL cholesterol but not LDL-C (19). The American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) recently updated their position statements on the management of T2D in adults to include additional focus on CVD risk factor management (4), but the recommendations for T1D are vague (4) as the ADA admits that very little clinical trial evidence exists for patients with T1D of any age to issue any meaningful recommendations (1).

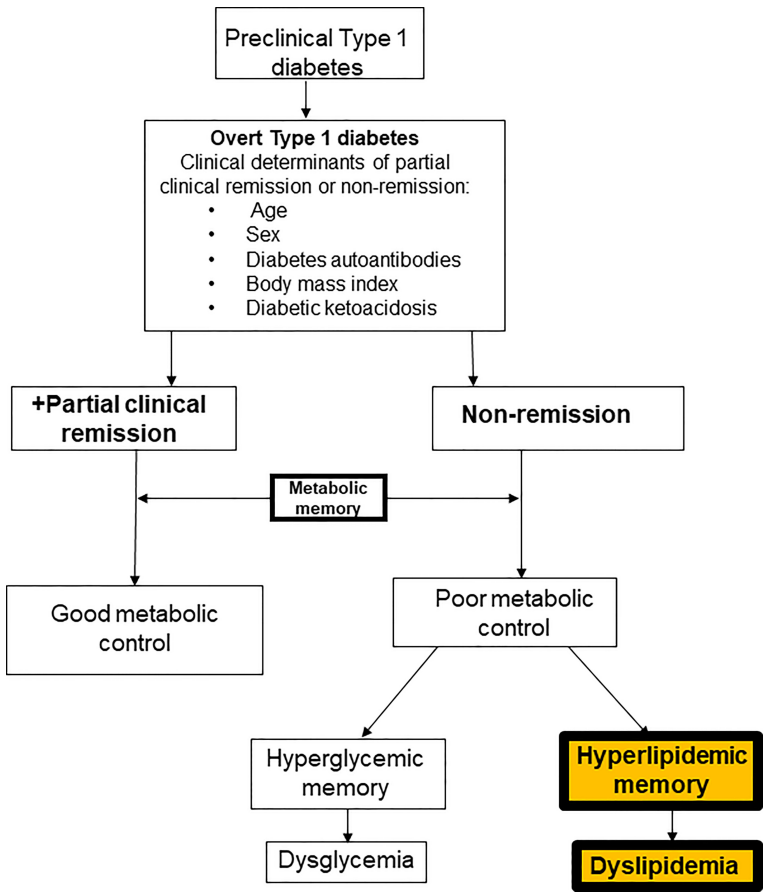

FIGURE 1 | A composite scheme of the dichotomy of subjects with type 1 diabetes based on the history of partial clinical remission into remitters and non-remitters and the role of metabolic memory on long-term metabolic parameters. The theory of *hyperglycemic* memory explains the dysglycemia in non-remitters while the theory of *hyperlipidemic* memory explains the dyslipidemia in non-remitters.

The current ADA guidelines (1), derived mainly from T2D data, recommend an initial screening for dyslipidemia in adults with diabetes mellitus at the time of diagnosis or at the initial clinic visit, and then to initiate interventions such as intensification of lifestyle modification and optimization of glycemic control for patients with elevated triglyceride levels (≥150 mg/dL) and/or low HDL cholesterol (<40 mg/dL for men, and <50 mg/dL for women). This is based on the concept of the atherogenic index of plasma, denoted by TG/HDL where an atherogenic index of >2 is related to CVD (20, 21).

C-Peptide Physiology in Relation to Partial Clinical Remission (PR) in Children With Type 1 Diabetes, and the Early Lipid Changes in Type 2 Diabetes Compared to Controls

There is no consensus on the mechanism of dyslipidemia in children with either T1D or T2D. Available reports have differed on the clinical patterns of dyslipidemia in both diseases, as well as the proposed mechanism(s) for the early-phase dyslipidemia in either disease. A key limitation of previous studies was the *lack of consideration* for the role of the honeymoon phase of T1D, also known as partial clinical remission (PR) on the early changes in lipid profile in patients with T1D despite reports that remission status confers special CVD risk protection to youth (6) and adults (8) with T1D. The principal marker for the honeymoon phase or PR is stimulated serum C-peptide concentration (22), and its clinical surrogate marker is the insulin-dose adjusted A1c (22), a functional marker that incorporates both A1c and total daily dose of insulin to determine PR status. C-peptide is a 31 amino acid peptide that is co-secreted with insulin (23). It's longer half-life of 31 minutes compared to 4 minutes for insulin, makes it a veritable tool for the confirmation of endogenous insulin production and secretion (24, 25). C-peptide has no known receptor, but has been reported to reduce diabetes-related complications such as neuropathy, retinopathy, nephropathy through various hypothesized mechanisms (23, 25–28) which are linked to its regulation of cell proliferation and apoptosis *via* its association with inflammatory mediators such as nuclear factor kappa B, tumor necrosis factor alpha, and protein kinase-C (29). However, the influence of C-peptide on the complications of either T1D or T2D through its impact on

early lipid changes in either T1D or T2D has not yet been fully investigated.

Type 1 Diabetes: The Limitations of the Current Hyperglycemic Theory

PR is a characteristic feature of T1D, which is a disorder of persistent hyperglycemia resulting from autoimmune destruction of the pancreatic β-cells (30, 31). PR often follows the diagnosis of T1D, and this phase is marked by an increased functionality of the surviving β-cells with attendant increased endogenous insulin production (32, 33). Subjects who experienced PR are designated as remitters and those who did not are designated as non-remitters. PR typically lasts for 3-12 months (22), however, recent studies have shown evidence for C-peptide production, and thus residual β-cell function, at more than 5 years following the diagnosis of T1D (34). During PR, C-peptide is co-secreted with insulin from pancreatic β-cells and this residual C-peptide may act as a surrogate marker of residual β-cell function. In physiologic concentrations, C-peptide acts to improve both microvascular blood flow and microvascular endothelial function through the release of endothelial nitric oxide (35). Following the diagnosis of T1D, serum C-peptide concentration undergoes an initial exponential fall followed by a stable phase of decline that may last for several years (34). The presence of residual endogenous insulin secretion in patients with T1D has been linked to reduced risk for severe hypoglycemia (36, 37), development of diabetic retinopathy (38), promotion of statural growth in prepubertal children (39) and a sustained improvement in long-term glycemic control (7, 8). Conversely, the non-remitters experience chronic hyperglycemia from the time of diagnosis (5, 7). This initial phase of chronic hyperglycemia has been associated with long-term complications of diabetes mellitus, regardless of whether glycemic control improved much later in the history of the disease (40). This phenomenology of diabetes complications arising from initial chronic hyperglycemia has been christened the theory of hyperglycemic memory (41). Recent studies show that there are non-glycemic aspects to this phenomenon, and most of these factors are yet to be fully characterized (40). As a result, some investigators now refer to this phenomenon as the glyco-metabolic theory (40). It is generally believed that the mechanisms that lead to the glyco-metabolic memory are *interdependent* and act simultaneously. The four mechanisms currently proposed are oxidative stress, generation of advanced glycation end-products, chronic inflammation, and epigenetic changes (40). ***However, none of the studies in this field has examined the initial post-diagnostic lipid phenotypes in these patients to determine whether a dichotomy exists in the lipid parameters, and whether non-remission is associated with both hyperglycemia and hyperlipidemia.*** Therefore, the theory of *hyperglycemic* memory has limited application as it does not explain the glycemic-independent dichotomy in early lipid phenotypes that presages subsequent differences in ASCVD risks and diabetes-related complications. A theory of *hyperlipidemic* memory, on the other hand, aptly explains this dichotomy and provides the necessary framework to understand the differences in lipid phenotypes between remitters and non-remitters on one hand, and between those with T1D or T2D on the other. This new paradigm is supported by a longitudinal study that reported a significantly reduced risk for chronic microvascular complications at 7-year follow up in young adults who experienced PR (7), as well as another study showing favorable lipid phenotype 5 years after the diagnosis of T1D in children who experienced PR (42).

Type 2 Diabetes

T2D, on the other hand, is a complex genetic disorder marked by persistent hyperglycemia as a result of a combination of increased β-cell apoptosis and insulin resistance (43, 44). There are significant pathophysiological, prodromal, and post-diagnostic differences between T1D and T2D that play important roles in their early lipid phenotypes. Sagesaka et al. reported that glucose dysregulation precedes the actual diagnosis of T2D by >10 years (45) in adults, while Lebovitz et al. reported that β-cell dysfunction in adults precedes clinical diagnosis of T2D by 12 years (46). In contrast, the diagnosis of T1D is often followed by the honeymoon phase or PR which largely determines the risks for early-phase dyslipidemia (6), mid-term microvascular disease risk (7), and long-term CVD risk (8). We have directly compared lipid-based CVD risk profile between T2D and T1D patients based on the PR history of the T1D cohort (5, 47).

Furthermore, the assumption that early dyslipidemia in children and adolescents with T2D is due to increased insulin resistance (IR) has not been tested by comparing their lipid parameters to those of non-remitting subjects with T1D, who do not have significant IR. Such a comparison will likely determine the role of IR on early lipid changes in children with diabetes mellitus; and may lead to a unified mechanistic model for dyslipidemia in those with diabetes mellitus. We have presented evidence from our studies showing that PR is the primary determinant of early lipid phenotype in pediatric and adult T1D, while other determinant such as BMI, sex, race/ethnicity, and glycemic control play only secondary roles (5, 6, 42, 48) as detailed below.

RATIONALE FOR INVESTIGATIONS ON THE THEORY OF HYPERLIPIDEMIC MEMORY IN PEDIATRIC TYPE 1 DIABETES: LACK OF CONSENSUS ON EARLY LIPID PHENOTYPES IN CHILDREN AND ADOLESCENTS

Background

Cardiovascular disease (CVD) is the leading cause of mortality in patients with diabetes mellitus (49). Dyslipidemia and atherosclerosis, which begin in childhood and adolescence[29,30], are primary contributors to the increased CVD risk in patients with T1D (50, 51). A pediatric study reported that 25% of youth with T1D have progressive and persistent dyslipidemia and

increased arterial stiffness (11), while another found a positive association between increased arterial stiffness and total cholesterol (TC), LDL-cholesterol (LDL-C), and HbA1c (12). There is, however, no consensus on either the patterns of early lipid changes, or the mechanism of these changes in children and adolescents with either T1D or T2D.

Lack of Consensus on the Patterns of Early Dyslipidemia in T1D and T2D

Hanks and co-workers conducted a comparative analysis of primary lipid parameters in overweight/obese youth with either T1D or T2D and found no significant differences in the concentrations of the primary lipid parameters between the groups (52). However, Rodriguez et al (53) reported a higher prevalence of CVD risk factors in youth with T2D compared to T1D, while Kim et al (54), in a 10-year longitudinal study that examined overall CVD risks between T1D and T2D reported increasing prevalence of elevated waist circumference in patients with T2D as its primary finding. Kim et al (54) found no significant longitudinal changes in the prevalence of other risk factors, including lipid concentrations, throughout the period of observation. Thus, there is no consistent pattern for early dyslipidemia in children and adolescents with T1D and T2D that could form the basis for a unified mechanistic theory of dyslipidemia in children with diabetes mellitus.

Lack of Consensus on the Mechanism of Early Dyslipidemia in T1D and T2D

There is equally no consensus on the mechanisms for early-phase dyslipidemia in youth with either T1D or T2D due to the disparate conclusions from published studies (55–59). Maahs et al. and other investigators had suggested that adiposity and IR (54, 58, 59) played a central role in the pathogenesis of dyslipidemia in children with diabetes mellitus. A cross-sectional study from the SEARCH Group (60) in the US also reported a relationship between increasing A1c and dyslipidemia in subjects with either T1D or T2D, but a UK-based longitudinal study in subjects with T1D found no such association (55). In support of the findings from the UK-based study (55), Katz et al, in a longitudinal retrospective cohort study of subjects with T1D found that changes in HbA1c and BMI z scores had minimal impact on LDL-C and non-HDL cholesterol (13). Though Shah et al (11, 13) and others reported a significant relationship between poor glycemic control and dyslipidemia in T1D (11, 13), others found an inconsistent pattern of correlation of lipid concentrations and HbA1c (61), or no correlation at all (62). Snell-Bergeon and others reported that systemic inflammation (57, 63) and glycemic control (55–57) play only a marginal role on early lipid changes in either T1D or T2D. Therefore, there is no consensus on early lipid phenotypes in children with diabetes mellitus. It is possible that a lack of consideration for the role of residual β-cell function or honeymoon phase in their respective T1D cohorts (52–54, 64) could have led to the disparate conclusions. None of the above studies explored the differences in lipid profiles based on patients' remission status, except in the case of Redondo et al (65) whose findings were confounded by the underestimation of PR by insulin dose adjusted A1c (IDAA1c) in ethnic minority youth (66).

Pathway to a Consensus on the Mechanism and Pattern of Early Dyslipidemia in T1D and T2D

The stratification of subjects with new-onset T1D by PR status is critical to ensure meaningful comparisons of lipid parameters for valid results (11–14). For instance, it is unknown whether the study that reported progressive and persistent dyslipidemia (11) contained a higher proportion of non-remitters, while the study that reported only a modest effect of HbA1c and BMI on lipid parameters (13) had a higher proportion of remitters. The fact that non-remitters make up >50% of children and adolescents with new-onset T1D (67, 68) makes it crucial to stratify subjects based on their PR history in all research studies assessing lipid parameters in patients with T1D. This will ensure proper stratification of risk for CVD by PR and may lead to the accumulation of data to designate non-remission as a non-modifiable risk factor for ASCVD in patients with T1D.

INVESTIGATION OF THE ROLE OF PARTIAL CLINICAL REMISSION ON EARLY LIPID PHENOTYPES IN PEDIATRIC TYPE 1 DIABETES

Clinical Studies Demonstrating the Role of PR on Early Lipid Phenotypes in T1D

To explore the role of PR on early lipid changes in children, we conducted a longitudinal retrospective cohort study of 123 children and adolescents with T1D of 5-year duration (6). The subjects' mean age was 11.9 ± 2.9 years, and the cohort consisted of 55 male subjects and 68 female subjects. There were 44 remitters and 79 non-remitters. A timeline of 4-5 years after diagnosis was chosen in concert with the American Diabetes Association (ADA) recommendation to initiate screening for diabetes complications in children either at the inception of puberty or 4-5 years after diagnosis (69) as it was previously believed that there was minimal risk of dyslipidemia during the prepubertal years (69). This study excluded children with dyslipidemia or a family history of lipid abnormalities. The results showed that children and adolescents who experienced PR had significantly lower mean LDL-C 4-5 years after the diagnosis of T1D compared to their peers who did not experience PR (6), after controlling for age, puberty, glycemic control, and adiposity [**Figure 2** (6)]. The significantly lower LDL-C in remitters was rather striking as a greater proportion of the remitters were in puberty 4-5 years after the diagnosis of T1D compared to the non-remitters. This was the first report to provide critical and objective evidence of an early lipid-based cardiovascular protection by PR in children with T1D.

To validate these results and confirm that these lipid-based findings were not influenced by normal, physiological, puberty-mediated changes in lipid concentrations in youth, we conducted

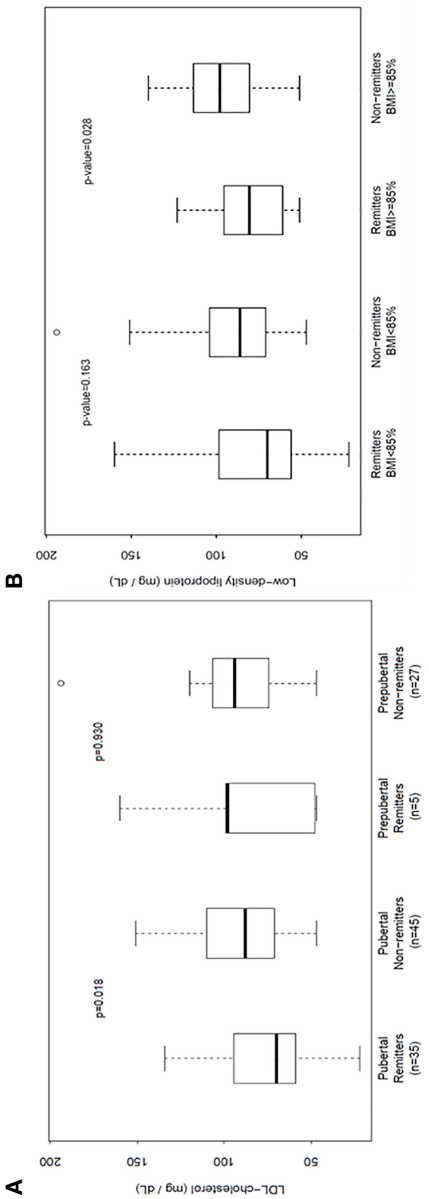

FIGURE 2 | **(A)** (left panel) and **(B)** (right panel): **(A)** shows that analysis of the differences in serum low-density lipoprotein cholesterol (LDL-C) concentration in the first 4-5 years of type 1 diabetes stratified by both pubertal and remission status. LDL-C concentration was similar in prepubertal remitters vs. non-remitters (p=0.930) but was significantly lower in remitters in puberty compared to the non-remitters in puberty (p=0.018) after adjusting for age and duration of diabetes (6). **(B)** depicts the analysis of the changes in serum low-density lipoprotein cholesterol (LDL-C) concentration in the first 4-5 years of type 1 diabetes stratified by both body mass index and remission status. Serum LDL-C was similar between the normal-weight remitters and non-remitters: 79.0 ± 32.8 mg/dL vs. 89.8 ± 27.5 mg/dL, p=0.17. In contrast, LDL-C was significantly lower in remitters among the overweight/obese cohort, 78.5 ± 21.1 mg/dL vs 95.6 ± 24.2 mg/dL, p=0.028 (6).

a follow-up study that compared the T1D cohort to age-matched controls.

INVESTIGATION OF THE ROLE OF PUBERTAL MATURATION ON EARLY LIPID CHANGES IN REMITTERS, NON-REMITTERS, AND CONTROLS

The primary rationale for the second study (8) was to determine whether pubertal maturation impacts physiological changes in lipids in children and adolescents with T1D by comparing the T1D cohort to controls to investigate whether subjects with T1D showed similar lipid changes as controls during puberty. This is crucial as the origins of the dichotomy in CVD risk in adults with T1D are rooted in childhood (6–8), but the exact mechanism and point of divergence from normal in CVD risk are not known. The secondary rationale was to either support or disprove the unverified hypothesis that youth with T1D did not experience a reduction in TC, LDL-C, and non-HDL during puberty (62), a phenomenon that occurs in healthy children and adolescents without T1D (70, 71).

This study (42) included 194 subjects consisting of 71 controls of age 12.9 ± 1.3y and 123 subjects with T1D stratified into remitters (n=44, age 13.0 ± 0.8y) and non-remitters (n=79, age 11.2 ± 0.6y). PR was defined as insulin-dose adjusted HbA1c of ≤9 (22). Pubertal status was determined by Tanner staging of breast development in girls, and testicular volume in boys. We found that among the pubertal cohort, LDL-C was significantly higher in the non-remitters compared to the remitters, 91.1 ± 25.6mg/dL vs 77.2 ± 25.8 mg/dL, p=0.018; and the normal-weight controls, 91.1± 25.6 mg/dL vs 70.4 ± 22.9 mg/dL, p=0.009; but was similar between the overweight/obese controls and non-remitters, 89.7 ± 28.9 mg/dL vs 91.1± 25.6 mg/dL, p=0.81, and similarly between the normal-weight controls and remitters, 70.4 ± 22.9 mg/dL vs 77.2 ± 25.8 mg/dL, p=0.39 [**Figure 3A** (42)]. Both non-HDL-C and TC showed similar patterns as the LDL-C.

This was the first study to characterize the natural pattern of lipid profiles in children and adolescents with T1D as they traverse through puberty based on stratification by remission status, while comparing their lipid profiles to healthy peers [**Figure 3B** (42)].

There were 3 novel findings from this study. The first finding was that remission status largely determines the pattern of lipid concentrations in youth with T1D during puberty such that children with T1D who experienced the honeymoon phase or PR showed similar reductions in LDL-C, TC, and non-HDL-C as do normal-weight, healthy children without T1D (71), while non-remitters did not. The study further showed that the *timing* of the onset of the *dichotomy* in lipid profiles, and consequent CVD risk, in youth with T1D occurs between ages *11-12* years for LDL-C, TC, and non-HDL cholesterol. This age definition for lipid phenotype dichotomy is consistent with the timing of the onset of physiologic reduction in LDL-C, TC, and non-HDL during puberty in children without diabetes mellitus (72).

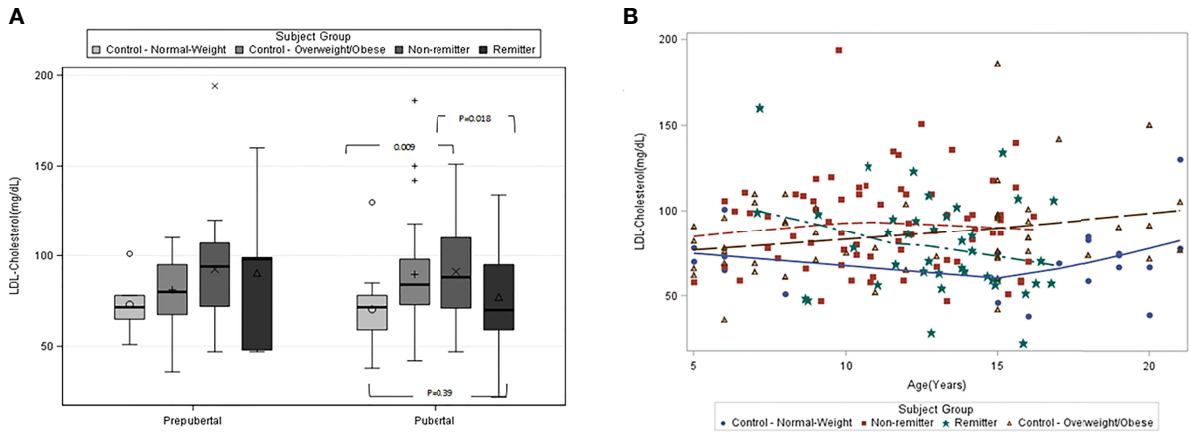

FIGURE 3 | **(A)** (left panel) and **(B)** (right panel): **(A)** is the box plots of low-density lipoprotein cholesterol (LDL-C) concentration stratified by pubertal status in controls and subjects with type 1 diabetes. Among the pubertal cohort, LDL-C was significantly higher in the non-remitters compared to the remitters (p=0.018), significantly higher in the non-remitters compared to the normal-weight controls (p=0.009). LDL-C was significantly higher in the overweight/obese controls compared to the normal-weight control (p=0.033), but similar between the normal-weight controls and remitters (p=0.39) (42). **(B)** is the scatterplot of the comparison of the patterns of low-density lipoprotein cholesterol (LDL-C) in controls and subjects with type 1 diabetes. Both the remitters and the normal-weight controls demonstrated lower LDL-C during puberty, while the overweight/obese controls and the non-remitters did not (42).

The stratification of the subjects into remitters and non-remitters was central to these findings, and thus showed that the lack of consensus on lipid phenotypes in children and adolescents with T1D from earlier studies could have derived from the non-stratification of subjects by PR history (11–14).

The second novel finding was that remitters have an intrinsic protection against adiposity-driven dyslipidemia, and this protection was absent in non-remitters as demonstrated by the significantly elevated LDL-C in overweight/obese non-remitters compared to overweight/obese remitters during puberty [**Figure 2** (6)]. This is consistent with the earlier report that residual C-peptide has a vascular protective function (8) and could protect remitters from early-phase anatomic changes in vasculature caused by dyslipidemia.

The third novel finding was that overweight/obese children without T1D do not experience the classic physiologic reduction in LDL-C, TC, and non-HDL that was described by Eissa et al (71) in healthy children and adolescents during puberty. This finding is important because Eissa et al (71) did not stratify their subjects by BMI status, and so were unable to detect this secondary effect that is largely driven by adiposity. The detection of a dichotomy in lipid phenotypes in normal children based on their BMI status led us to the hypothesis that the increased levels of LDL-C, non-HDL, and TC in the overweight/obese children and adolescents might be due to the presence of non-functional C-peptide in their circulation, similar to the concept of insulin resistance.

This study highlights the central role of C-peptide physiology on early lipid changes in children and adolescents. It is generally believed that the mechanism for the reduction in LDL-C, TC, and non-HDL-C during puberty is related to the effect of sex hormones on lipoprotein metabolism, specifically changes in alpha and beta lipoproteins (72). We proposed that this puberty-mediated reduction in the concentrations of LDL-C, TC, and non-HDL-C could be attenuated or abolished by increased insulin resistance (73) as reported in our overweight/obese cohort, due to the non-functional C-peptide effect. In contrast, PR appears to facilitate this normal physiologic reduction in LDL-C, TC, and non-HDL-C in youth with T1D. This reduction is however absent in the non-remitters, who lack endogenous insulin or C-peptide activity. This concept of non-functional C-peptide effect was recently confirmed by Mock et al (74) who reported that 55% of youth with new-onset T1D and detectable C-peptide of >300 pmol/L had low insulin sensitivity scores at 14.5 months following the diagnosis of T1D, and thus were not in PR when defined by IDAA1c.

Based on these finding we decided to explore whether a C-peptide mechanistic model or an adiposity model (based on BMI) could explain early changes in lipids in T2D by comparing subjects with T2D, who are classically insulin resistant, to non-remitters who are relatively not insulin resistant, while using the controls and remitters as comparators.

A COMPARATIVE ANALYSIS OF THE EARLIEST POST-DIAGNOSTIC LIPID PHENOTYPES IN REMITTERS, NON-REMITTERS, TYPE 2 DIABETES, AND CONTROLS IN CHILDREN AND ADOLESCENTS

The primary rationale for this investigation of the early lipid phenotypes in children and adolescents with either T1D or T2D was to explore the basis for the lack of consensus on the accurate patterns and mechanisms of early ASCVD risk in children and

adolescents with either T1D or T2D (52–54, 64). The secondary rationale was to investigate the unproven premise that pediatric patients with T2D have worse lipid profiles than their peers with T1D in the early phases of T1D or T2D. This is important as no prior study had compared early lipid phenotypes in patients with either T1D or T2D after stratifying the T1D cohort into remitters and non-remitters despite reports that remission status confers special CVD risk protection on youth (6) and adults (5) with T1D.

The aim of this investigation was to determine the differences in ASCVD risk, using lipid parameters as surrogates, in children and adolescents with either T1D or T2D at the time of their first lipid assessment, after stratifying the T1D cohort into remitters and non-remitters. The study's hypothesis was that the remitters and controls would have similar and more favorable lipid phenotype compared to the non-remitters and subjects with T2D.

This study (47) included 249 subjects of <21 years consisting of 73 controls, 53 T2D subjects, and 123 T1D subjects stratified into remitters (n=44), and non-remitters (n=79). Partial clinical remission (PR) was defined as insulin-dose adjusted HbA1c of ≤9, and pubertal status was determined by Tanner staging of breasts in girls and testicular volume in boys [**Table 1** (47)]. The results showed that after adjusting for age, sex, BMI, race, and pubertal status, patients with T2D had significantly higher LDL-C compared to the controls (103.1 ± 5.9 mg/dL vs 83.9 ± 3.6 mg/dL, p=0.022), the remitters (103.1 ± 5.9 mg/dL vs 79.1 ± 5.2 mg/dL, p = 0.029), but not the non-remitters (103.1 ± 5.9 mg/dL vs 91.4 ± 4.2 mg/dL, p = 0.49) [**Figure 4** (47)].

Similarly, T2D patients had significantly higher non-HDL-C compared to the controls (p=0.006), the remitters (p=0.0002), but not to the non-remitters (137.6 ± 7.1 mg/dL vs 111.71 ± 5.0 mg/dL, p=0.053). Total cholesterol was also significantly higher in T2D patients compared to the controls (p=0.0005), the remitters (p=0.006) but not to the non-remitters (183.5 ± 6.6 mg/dL vs 166.2 ± 4.8 mg/dL, p=0.27).

This study showed that after adjusting for confounding variables, the serum concentrations of the primary lipid markers: LDL-C, non-HDL-C, and TC were significantly elevated in children and adolescents with either T2D or the non-remitters, compared to controls and the remitters. This report, which is based on stratification of T1D subject into remitters and non-remitters, *clarifies* the long-standing incongruent results of earlier studies that evaluated lipid phenotypes in children with either T1D or T2D (52–54, 64), and makes the case for the stratification of subjects with T1D into remitters and non-remitters to ensure valid comparisons of early lipid phenotypes in this field.

A COMPARATIVE ANALYSIS OF THE EARLIEST POST-DIAGNOSTIC LIPID PHENOTYPES IN REMITTERS, NON-REMITTERS, TYPE 2 DIABETES, AND CONTROLS IN ADULTS

The rationale for this investigation of the early lipid phenotypes in adults with either T1D or T2D was predicated on the fact that risk factors for ASCVD are well established in T2D (1), but not in T1D (3, 4). This is an important area of study as no prior study in adults has compared early lipid phenotypes in patients with either T1D or T2D after stratifying the T1D cohort into remitters and non-remitters, despite reports that remission status confers special CVD risk protection on patients with T1D (6, 8).

The aim of this study was to investigate the impact of PR on the earliest ASCVD risk phenotype in adult patients with T1D by using factor analysis to quantify and compare the ASCVD risk

TABLE 1 | Anthropometric and biochemical characteristics of the subjects (47).

Parameters	Controls n=73	Non-Remitters n=79	Remitters n=44	Type 2 diabetes n=53	*p* value
Age (years)	12.8 ± 5.2	11.3 ± 2.9	13.0 ± 2.5	18 ± 3.1	<0.001
Sex					0.346
• Male (%)	53%	41%	52%	43%	
• Female (%)	47%	59%	48%	57%	
Race					0.001
• White (%)	62%	78%	82%	51%	
• Non-white (%)	38%	22%	18%	49%	
Pubertal Status					<0.001
• Tanner I (%)	37%	38%	14%	0%	
• Tanner II-V (%)	63%	62%	86%	100%	
BMI Status in percentile					<0.001
• Normal-weight (<85th) (%)	29%	70%	64%	0%	
• Over-weight/obese (≥85th) (%)	71%	30%	36%	100%	
Height z-score	0.2 ± 1.4	-.01 ± 1.2	0.1 ± 0.9	0.9 ± 1.4	<0.001
Weight z-score	1.6 ± 1.3	0.5 ± 1.0	0.7 ± 0.8	2.7 ± .7	<0.001
(BMI) z-score	1.7 ± 1.1	0.63 ± 0.9	0.7 ± 0.8	2.4 ± .4	<0.001
SBP (mm Hg)	111.7 ± 11.8	107.8 ± 11.8	111.3 ± 12.8	122.4 ± 13.1	<0.001
DBP (mm Hg)	69.9 ± 8.9	70.2 ± 7.0	70.6 ± 6.0	76.7 ± 8.1	<0.001
Hemoglobin A1c (%)	N/A	8.8 ± 1.2	8.6 ± 1.5	6.7 ± 1.3	<0.001

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. N/A, not applicable. Remission status was defined by an insulin-dose adjusted hemoglobin A1c (IDAA1c) of ≤9 (22).

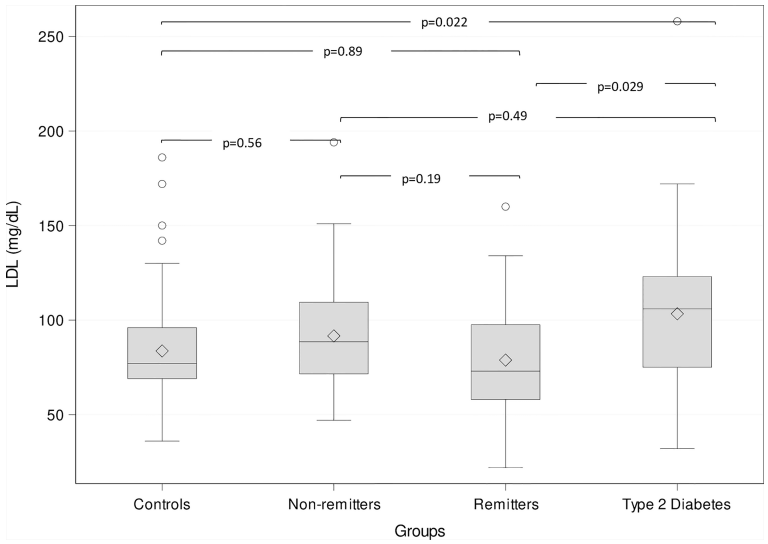

FIGURE 4 | Box plots of the comparison of serum low-density lipoprotein (LDL)-cholesterol (LDL-C) among the groups. The lines through the middle of the boxes represent the median or middle quartile, while the lines at the top and bottom of box represent the upper and lower quartiles, respectively. The upper and lower whiskers represent the scores outside the middle 50%, while the circles represent the outliers. Serum LDL-C was significantly higher in the subjects with type 2 diabetes compared to the controls and the remitters, but similar to the non-remitters (47).

scores of lipid phenotypes in T1D, T2D and the controls after stratifying the T1D cohort into remitters and non-remitters. We focused this aim primarily on the factor analysis of the American Diabetes Association-recommended initial lipid parameters for the assessment of CVD risk in adults with diabetes namely, TG, HDL-C, and the atherogenic index of plasma, TG/HDL as factor 2; and secondarily on non-HDL-C, LDL-C, TC, TC/HDL-C ratio as factor 1. We hypothesized that the remitters and controls would have similar and more favorable lipid phenotype compared to the non-remitters and subjects with T2D. We further speculated that a proof of this hypothesis could lead to investigations that might generate a generalized theory for the earliest mechanisms of atherogenic lipid profile in patients with either T1D or T2D.

This was a study of 203 subjects consisting of 40 controls, 77 subjects with T1D, and 86 subjects with T2D. The subjects with T1D were further divided into remitters (n=49) and non-remitters (n=28). The overall mean age was 37.3 (± 12.7 SD), with male subjects 51.7% and white subjects 71.3%. Subjects were excluded if they had dyslipidemia, family history of dyslipidemia, or were receiving lipid-lowering medications. **Table 2** (5) shows the baseline anthropometric and biochemical characteristics of the subjects by study group. There were no significant differences in height or gender distribution between the remitters, non-remitters, and subjects with T2D (p=0.44 and 0.91, respectively). Subjects with T2D were older, heavier, and had higher systolic and diastolic blood pressure readings than the subjects with T1D (p<.0001). The non-remitters had significantly higher fasting blood glucose levels (p<.0001). **Figure 5** [original data from Nwosu et al (5)] shows the pattern of glycemic control in the subjects in the first year of the study. The non-remitters had the worst glycemic control in the 12 months of observation.

Lipid Analysis

Individual Lipid Parameters and Ratios

The initial analysis examined the differences in the individual lipid parameters and ratios among the controls, remitters, non-remitters, and T2D subjects. For the individual lipid parameters, the median and the first and the third quartiles were reported to address the skewed distribution of these parameters.

Non-HDL-C

Serum non-HDL-C was significantly lower in the controls [median=100 mg/dL, Q1-Q3= (84-116)] compared to the subjects with T2D (152 mg/dL, 119-179, p<0.0001), and the non-remitters (131 mg/dL, 100-167, p<0.0001), but was similar to the remitters (116 mg/dL, 92-155, p=0.051). Additionally, non-HDL-C was significantly lower in the non-remitters compared to the subjects with T2D (p=0.027) but was similar between the remitters and non-remitters (p=0.39) [**Figure 6** (5)].

TG

Serum TG concentration was significantly lower in the controls (69 mg/dL, 50-88) compared to the subjects with T2D (194 mg/dL, 134-276, p<0.0001) but was similar between the remitters and non-remitters (94 mg/dL, 66-157 vs 107 mg/dL, 82.5-184, p=NS). Though TG was similar between the non-remitters and subjects with T2D (p=NS), it was significantly lower in the remitters compared to the subjects with T2D (p<0.0001).

TG/HDL-C

TG/HDL-C ratio was significantly lower in the controls compared to the subjects with T2D (1.2, 0.9-1.7 vs 5.7, 3.1-8, p<0.0001), the non-remitters (1.2, 0.9- 1.7 vs 2.4, 1.5-4.9, p=0.003), but similar to the remitters (1.2, 0.9-1.7 vs 1.8, 1.2-3.3, p=NS). Furthermore, TG/

TABLE 2 | Comparison of anthropometric, biochemical, and therapeutic parameters (5) .

Parameters	Controls (n=40)		Remitters (n=49)		Non-remitters (n=28)		Type 2 diabetes (n=86)		ANOVA F-test p value	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	All 4 groups	3 DM groups
Age (year)	33.8	11.0	29.7	10.9	31.9	11.0	45.0	10.5	<.0001	<.0001
Height (cm)	164.9	9.5	170.9	11.8	167.1	8.8	170.4	10.2	0.0450	0.44
Weight (kg)	74.8	20.2	78.6	17.4	68.6	11.3	104.7	28.7	<.0001	<.0001
BMI (kg/m^2)	26.6	6.1	27.0	6.3	25.6	3.2	35.4	9.5	<.0001	<.0001
SBP (mm Hg)	114.6	17.6	117.5	13.0	113.3	14.0	133.3	17.3	<.0001	<.0001
DBP (mm Hg)	71.4	13.9	74.5	8.2	70.7	10.7	83.5	10.8	<.0001	<.0001
FBS (mg/dL)			224	130	425	232	201	116		<.0001
TC (mg/dL)	155.8	18.6	182.6	72.1	186.2	49.1	192.5	44.9	0.0014	0.62
LDL-C (mg/dL)	86.1	17.9	100.5	34.8	105.1	32.1	110.9	35.0	0.0011	0.31
HDL-C (mg/dL)	55.6	12.4	50.5	13.8	46.7	12.8	38.8	9.8	<.0001	<.0001
TC/HDL	2.9	0.7	4.1	3.9	4.2	1.6	5.2	1.7	<.0001	0.0329
Non-HDL-C (mg/dL)	100.1	20.3	132.1	74.3	139.5	48.6	153.7	45.6	<.0001	0.11
TG (mg/dL)	70.4	26.5	120.4	86.0	171.1	168.1	256.6	277.2	<.0001	0.0095
TG/HDL	1.3	0.6	2.7	2.3	4.2	4.7	7.6	9.5	<.0001	0.0041
HbA1c at 0 mo (%)			11.6	2.4	11.7	2.5	8.8	2.3		<.0001
HbA1c at 6 mo			6.5	0.9	9.0	2.1	7.1	1.4		<.0001
HbA1c at 12 mo			6.8	1.3	9.4	2.4	7.4	1.7		<.0001
TDD at baseline(units/kg/day)			0.39	0.18	0.51	0.29	0.40	0.18		0.15
TDD at 6 mo			0.39	0.20	0.70	0.35	0.27	0.19		<.0001
TDD at 12 mo			0.42	0.21	0.81	0.31	0.33	0.29		<.0001
Metformin (mg) baseline							1069	501		
Metformin (mg) final							1492	548		
	n	%	n	%	n	%	n	%		
Sex										
Male	12	30.0	28	57.1	15	53.6	50	58.1	0.0224	0.91
Female	28	70.0	21	42.9	13	46.4	36	41.9		
Race/Ethnicity										
White	26	65.0	44	91.7	18	64.3	56	65.1	0.0051*	0.0022*
Black	6	15.0	1	2.1	1	3.6	10	11.6		
Asian	3	7.5	0	0.0	1	3.6	4	4.7		
Hispanic	5	12.5	2	4.2	8	28.6	14	16.3		
Other	0	0.0	1	2.1	0	0.0	2	2.3		

*BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HbA1c, hemoglobin A1c; mo, month; TDD, total daily dose of insulin. *p value for white versus others*

HDL was significantly lower in the remitters compared to the non-remitters (p=0.007) [**Figure 7** (5)].

LDL-C

There was a significant difference in serum LDL-C among the 4 groups (p<0.0005). *Post hoc* analysis showed no differences in LDL-C levels among the remitters, non-remitters, and subjects with T2D. However, when compared to the controls, LDL-C was significant higher in the subjects with T2D (p<0.0004), non-remitters (p=0.009), but similar to the remitters (p=0.052).

HDL-C

Serum HDL-C was significantly lower in the subjects with T2D compared to the controls (52.5 mg/dL, 45.5-67.0 vs 36 mg/dL, 31.0-45.0, p<0.0001), non-remitters (52.5 mg/dL, 45.5-67.0 vs 49.5 mg/dL, 34.5-56.0, p=0.0217), and remitters (52.5 mg/dL, 45.5-67.0 vs 47.5, 42.0-62.0, p<0.0001). HDL-C was similar between the non-remitters and remitters (49.5 mg/dL, 34.5-56.0 vs 47.5 mg/dL, 42.0-62.0, p=NS)

TC/HDL-C

TC/HDL-C ratio was significantly lower in the controls compared to the subjects with T2D (2.9, 2.3-3.5 vs 5.1, 4.0-6.1, p<0.0001), and the non-remitters (2.9, 2.3-3.5 vs 3.8, 3.1-4.9, p=0.003), but was similar to the remitters (2.9, 2.3-3.5 vs 3.3, 2.7-4.3, p=NS). Additionally, TC/HDL-C was significantly lower in the remitters compared to the non-remitters 3.3, 2.7-4.3 vs 3.8, 3.1-4.9, p=0.026 [**Figure 8** (5)].

Factor Analysis of Lipid Parameters

Next, we employed factor analysis to confirm our findings in the individual lipid parameters and to stratify the groups based on their ASCVD risk potential by assigning composite risk scores to the factorized lipid parameters. Factorization of the 7 lipid parameters [**Table 3** (5)] yielded 2 orthogonal factors that jointly explained 89.5% of the total variance in the original 7 lipid parameters with their communalities ranging from 0.74 to 0.99. Based on the structure of the first factor, a composite score was calculated for each subject as a weighted sum of standardized values of the original 7 lipid parameters, with much heavier weights on TC and LDL-C. This composite score was named as TC*LDL.

The factor analysis demonstrated a linear increase in the means of both factor 1 (TC*LDL) and factor 2 (HDL*TG) composite scores from the control group to the remitters, non-remitters, and subjects with T2D, p value 0.0042, and <0.0001 respectively as shown in **Figures 9** and **10** (5). This is further

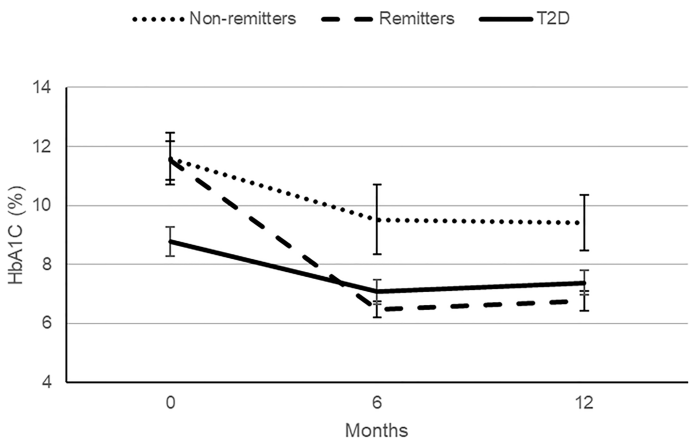

FIGURE 5 | A comparison of the patterns of glycemic control in the remitters, non-remitters, and subjects with type 2 diabetes (T2D) in the first 12 months following the diagnosis of diabetes mellitus [original data from Nwosu et al. (5)].

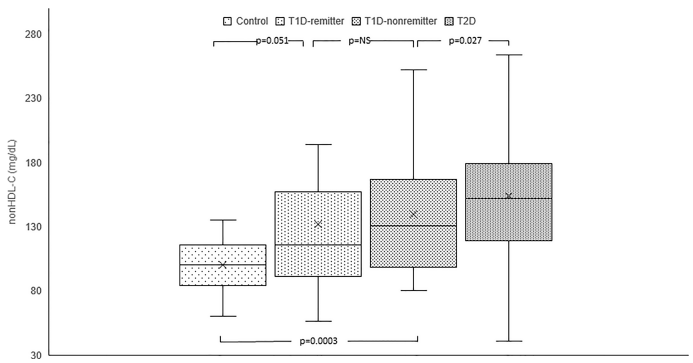

FIGURE 6 | Box plots of early post-diagnostic patterns of non-high density lipoprotein cholesterol (non-HDL-C) in the remitters, non-remitters, and subjects with type 2 diabetes (T2D) compared to controls. The box represents the 50th percent interquartile range, while the 'x' represents the mean and the horizontal line within the box represents the median, and the upper and lower whiskers represent 25th percentile above and below the mean, respectively (5).

illustrated in **Figure 11** (5), a composite two-dimensional plot of factor 1 and factor 2 showing that the controls and remitters occupy the low-risk quadrant, while the non-remitters and subjects with T2D occupy the higher-risk quadrants. These findings in adults confirmed our earlier results in children and adolescents and establish the phenomenon of early dichotomy in lipid parameters in patients with T1D, which we believe, presage the eventual dichotomy in ASCVD risk and prevalence in patients with T1D.

DISCUSSION

The Case for PR-Mediated Hyperlipidemic Memory as the Primary Determinant of Early Phenotypes in Both Pediatric and Adult T1D

A comprehensive analysis of the risk factors for dyslipidemia is crucial to the understanding of the central role of hyperlipidemic memory on early lipid phenotypes of both T1D and T2D in relation to other factors associated with dyslipidemia such as glycemic control, BMI, and insulin resistance. The role of glycemic control was examined by Nwosu et al (6) who showed that both remitters and non-remitters have poor glycemic control at the time of diagnosis of T1D, but that glycemic control improves markedly in the remitters and less so in the non-remitters, suggesting that poor glycemic control could lead to dyslipidemia in these patients. However, the fact that the T2D subjects in the follow-up study (47), with less favorable lipid parameters at the time of the diagnosis, had significantly lower mean A1c level of 6.7% compared to the T1D cohort (8.8% for the non-remitters, and 8.6% for the remitters) argues against glycemic control as the principal determinant of early-phase dyslipidemia in children with either T1D or T2D. This finding and previous reports (40) reflect a fundamental limitation of the theory of *hyperglycemic* memory to explain the dichotomy in lipid phenotypes in T1D. Additionally, though BMI is a predictor of dyslipidemia, the presence of normal BMI z-scores in the non-

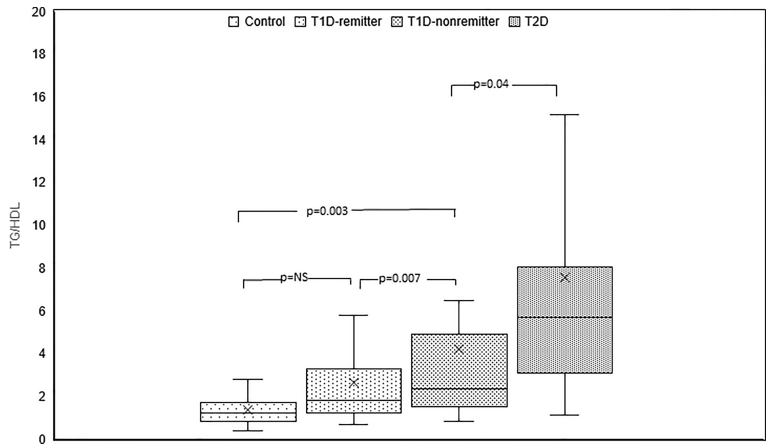

FIGURE 7 | Box plots of early post-diagnostic patterns of triglycerides/high-density lipoprotein cholesterol ratio (TG/HDL) in the remitters, non-remitters, and subjects with type 2 diabetes (T2D) compared to controls (5).

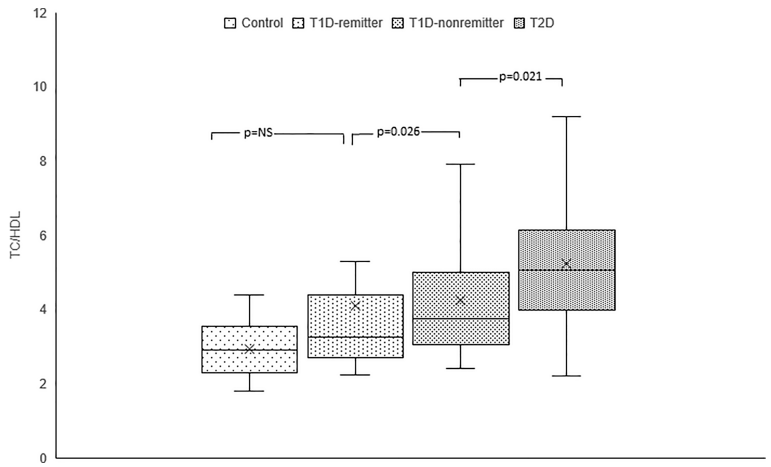

FIGURE 8 | Box plots of early post-diagnostic patterns of total cholesterol/high-density lipoprotein cholesterol ratio (TC/HDL) in the remitters, non-remitters, and subjects with type 2 diabetes (T2D) compared to controls (5).

TABLE 3 | Factor analysis of individual lipid parameters and ratios (5).

Factor name	Factor 1 TC*LDL	Factor 2 HDL*TG	Communality
TC	0.98	0.12	0.98
LDL	0.97	0.06	0.94
Non-HDL	0.92	0.39	0.99
TC/HDL	0.55	0.78	0.91
HDL	0.08	-0.86	0.74
TG	0.31	0.84	0.80
TG/HDL	0.19	0.94	0.91
%Variance explained	45.3%	44.1%	89.5%

Factors 1 and 2 were derived from factor loading with varimax rotation after adjusting for age, sex, body mass index and ethnicity. TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

remitters (BMI z-score of 0.63 ± 0.9), despite having a similar lipid profile as the obese T2D patients with a BMI z-score of 2.4 ± 0.4, suggests that increased BMI alone is not the primary etiological factor for increased dyslipidemia in the early phase of T1D or T2D in children. This is further supported by an analysis of the proportion of subjects with dyslipidemia (75) in that study that showed that LDL-C of >130 mg/dL occurred in 7 (13.2%) of the T2D subjects; 6 (7.6%) of the non-remitters; 2 (4.6%) of the remitters; and 4 (5.5%) of the controls. Similarly, TC of >200 mg/dL occurred in 15 (28.3%) T2D subjects; 9 (11.4%) non-remitters; 3 (6.8%) remitters; and 4 (5.5%) controls. This analysis suggests that the non-remitters and the subjects with T2D had a *higher frequency of dyslipidemia* compared to the remitters and controls. Finally, the similarity of early lipid profiles in patients with T2D

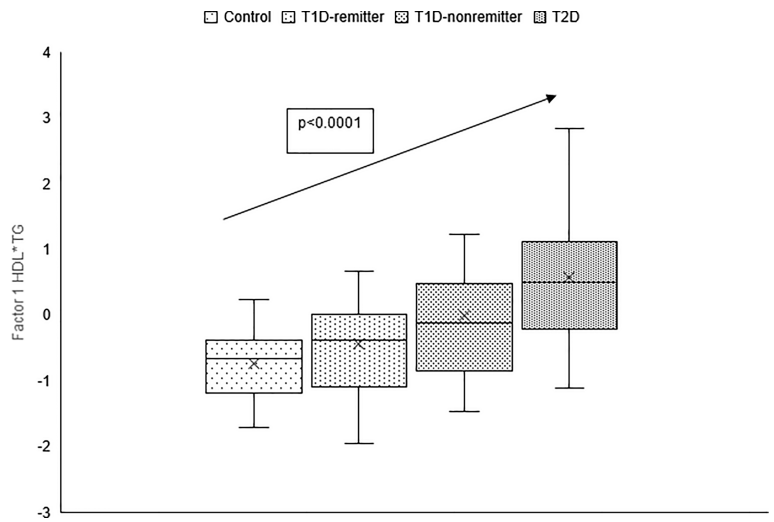

FIGURE 9 | Box plots of the factorial analysis of non-HDL-C, LDL-C, TC, TC/HDL ratio designated as summary factor 1 (TC*LDL) obtained with the factor loading threshold of ≥0.45 in 203 adults. Factor 1 explained 90% of the variance in the original lipid parameters with a linear increase in mean composite scores from controls, remitters, non-remitters, and subjects with type 2 diabetes (p=0.0042) (5).

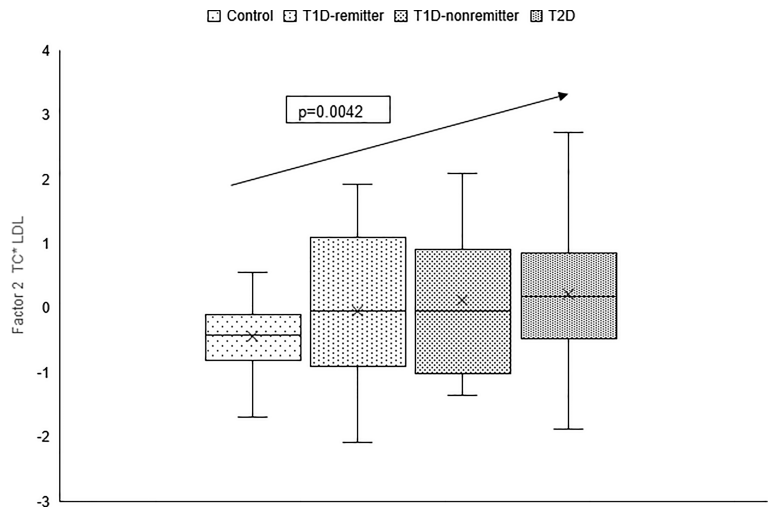

FIGURE 10 | Box plots of the factor analysis of the American Diabetes Association-recommended initial lipid parameters for the assessment of CVD risk in adults with diabetes namely, TG and HDL, and the atherogenic index of plasma, TG/HDL as designated as summary factor 2 (HDL*TG) obtained with the factor loading threshold of ≥0.45 in 203 adults. Factor 2 explained 90% of the variance in the original lipid parameters with a linear increase in mean composite scores from controls, remitters, non-remitters, and subjects with type 2 diabetes (p=0.0001) (5).

and the non-remitters, despite their significant differences in BMI z scores, also argues against IR as the sole driving force for dyslipidemia in non-remitters compared to the subjects with T2D. These findings which were confirmed in our adult study (5) establish PR as the primary determinant of early lipid phenotypes in both pediatric and adult T1D.

An alternative theory however could also be entertained. Though the above conclusions are supported by the study data in children and adults, it is possible to advance an alternative conclusion which proposes that the observed differences in the lipid profiles arose from differences in the degree of insulin resistance (IR) in each group such that partial clinical remission served only as a surrogate marker of IR. This conclusion is pertinent as IR occurs in both T1D (74, 76) and T2D; and a recent study by Mock et al. reported that 55% of subjects with new-onset T1D and detectable stimulated C-peptide level of >300 pmol/L had low insulin sensitivity (i.e., high IR) and thus were not in remission when assessed by insulin-dose adjusted

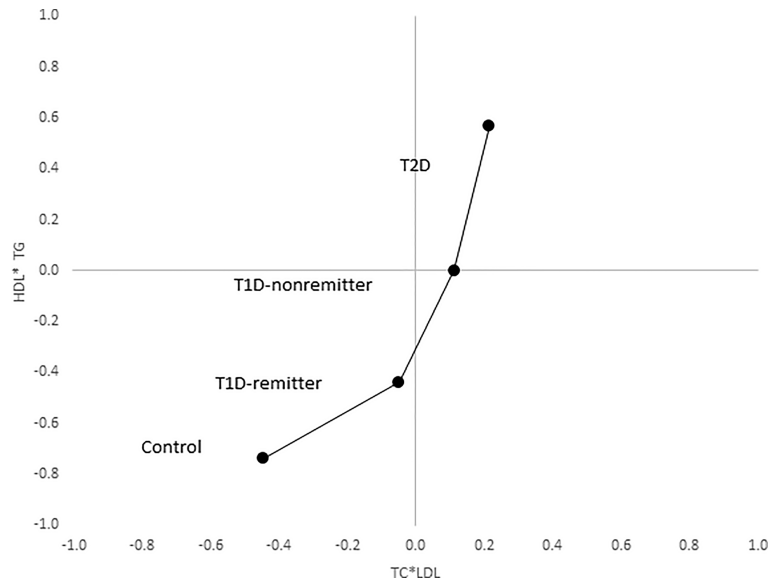

FIGURE 11 | Mean composite score for lipid parameters by factor analysis. Two-dimensional depiction of the mean composite scores of factor 1 (TC*LDL) and factor 2 (HDL*TG) from the factorial analysis of the 7 lipid parameters. Both the controls and remitters are in the low composite risk quadrant, whereas non-remitters and remitters are in the higher risk quadrants. Both factor 1 and factor 2 explained 90% of the variance in the original lipid parameters, p values 0.0042 and <0.0001 respectively. The p-values for linear trends were obtained from linear regression models on composite scores, adjusted for age, sex, ethnicity and BMI (5).

A1c (74). Therefore, partial clinical remission, in this alternative theory, may be a marker of IR, with remitters having low IR, and non-remitters having high IR similar to the high IR state in T2D.

Hyperlipidemic Memory and C-Peptide Physiology: The Synergistic Role of Insulin Sensitivity and C-Peptide Physiology on Early Lipid Phenotypes in Both T1D and T2D

Though C-peptide is considered as an indicator of preserved residual β-cell function, it is a metabolically active molecule (77). Our data suggest that the primary factor leading to the elevations in LDL-C, TC, and non-HDL-C in children and adults with either T1D or T2D is the absolute lack, or the functional absence of the protective role of C-peptide on early lipid changes in diabetes mellitus, such that in the early phases of T1D or T2D in children, there is a functional absence of endogenous C-peptide action in T2D, undetectable C-peptide action in non-remitters, but an active C-peptide effect in the remitters who still produce biologically-active, endogenous C-peptide. Though BMI contributes to dyslipidemia, it does not explain the similarity in lipid phenotypes between the non-remitters who are not obese, and the subjects with T2D, who are obese. However, given the recent study by Mock et al (74) reporting variable levels of insulin sensitivity in youth with similar C-peptide concentrations, it appears that the differences in lipid phenotypes in the various groups could result from a combination of functional C-peptide physiology and differences in insulin sensitivity. Data from our 4 studies suggest that insulin sensitivity and C-peptide physiology articulate a unified mechanistic model for early dyslipidemia across the lifespan in both children and adults with diabetes mellitus.

Strengths and Limitations of the Studies

The limitations of our studies are related to their retrospective design from which one cannot infer causality, as well as the relatively small sample size for the individual subgroups. The strengths of the study include the careful stepwise progression of the four investigations, the inclusion of pediatric and adult controls and subjects with T2D to clarify the dichotomy in lipid phenotypes in subjects with T1D, the inclusion of longitudinal data, the confirmation of the studies in children with a robust study in adults, and the establishment of composite risk scores for ASCVD by factor analysis.

Conclusions

We have presented evidence in support of the Theory of Hyperlipidemic Memory based on a series of 4 research studies. These studies which stratified subjects with T1D into remitters and non-remitters and compared their early lipid phenotypes to their peers with T2D as well as controls, demonstrated that across the lifespan: children, adolescents, and adults with T2D and their peers with T1D with no history of PR (i.e., non-remitters) have less favorable lipid phenotype compared to the remitters and controls. These findings strongly suggest the presence of a dichotomy in ASCVD *risk* in subjects with T1D, such that non-remitters have a higher composite risk score for lifelong ASCVD compared to the remitters. The confirmation of this dichotomy in lipid phenotypes between the remitters and non-remitters across the lifespan supports the theory of hyperlipidemic memory whereby the initial

hyperlipidemia in non-remitters persists across the lifespan leading to increased risk for ASCVD in this sub-population of subjects with T1D; whereas the imprimatur of PR in the remitters presages a lifetime of favorable lipid profile which has been confirmed in large studies (8). The concept of imprimatur of PR is apt as the metabolic advantages of PR continue long after the end of remission (7). This theory of hyperlipidemic memory explains the principal role of PR history on the early dichotomy in lipid phenotypes in T1D, the subsequent dichotomy in lipid-based ASCVD risks and may provide a new foundation for an early and accurate quantification of ASCVD risk in subjects with T1D across the lifespan.

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 Institutional Review Board of the University of Massachusetts Medical School. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

BN conceived the idea and wrote the manuscript. He is guarantor of the manuscript.

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Case report: 11-ketotestosterone may potentiate advanced bone age as seen in some cases of Wiedemann-Steiner Syndrome

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Context: Wiedemann-Steiner Syndrome (WSS) is a genetic disorder associated with an array of clinical phenotypes, including advanced bone age and short stature. 11-ketotestosterone (11KT) is a member of the group known as 11-oxygenated C19 androgens that are implicated in premature adrenarche.

Case description: Case 1: The patient is a 3 year and 11-month-old female diagnosed with WSS due to deletion of *KMT2A* detected on CGH microarray. At two years and 11 months, imaging revealed an advanced bone age. We obtained an 11KT level on this patient. 11KT in case 1 was elevated at 26.3 ng/dL, while the normal reference range is 7.3-10.9 ng/dL and the reference interval for premature adrenarche is 12.3-22.9 ng/dL, The repeat 11KT at follow up (chronological age 4 years and 6 months) was still elevated at 33.8 ng/dL Case 2: A second child with WSS and a 5kb intragenic *KMT2A* deletion was evaluated at 11 months of age; his 11KT was 4.5 ng/dL.

Conclusions: The elevated 11KT may indicate maturational changes related to increasing adrenal gland androgenic activation and may explain the advanced bone age seen in some patients with WSS. To our knowledge, this is the first case report that describes 11KT as a bioactive androgen potentially causing bone age advancement in WSS. Lack of elevation of 11KT in the second child who is an infant suggests increasing androgenic precursors and metabolites related to premature adrenarche may need to be longitudinally followed.

KEYWORDS

11-Ketotestosterone, Advanced Bone Age, Wiedemann-Steiner Syndrome, adrenarche, adrenal steroidogenesis

Introduction

Wiedemann-Steiner Syndrome (WSS) is a rare autosomal dominant condition first described by Wiedemann et al. in 1989 and expanded by Steiner and Marques in 2000 (1–3). Common findings include intellectual disability, dysmorphic facial features, short stature, developmental delay, and hypertrichosis including hypertrichosis cubiti (4, 5). WSS is associated with mutations in the *KMT2A* gene which encodes a histone methyltransferase that has a regulatory role in gene expression (6).

A consistent finding in WSS is dysregulated bone age, including previous reports of both delayed and advanced bone age (7). Bone age is most often measured through radiographs of the left hand and wrist or knee and scored with standardized methods such as Greulich and Pyle (8). Several case reports and cohort studies have identified individuals with WSS who also display an advanced bone age (4, 7, 9, 10). Advanced bone age is defined as a bone age that is two or more standard deviations above chronological age (11).

In general, causes of advanced bone age include familial constitutional advancement, sex steroid exposure, obesity, precocious puberty, tumors, adrenal disease, hyperthyroidism, and overgrowth syndromes (8). The mechanism behind WSS and bone age advancement remains unclear (12).

The main sex steroids that are known to have an influence on bone age are estrogens and androgens (13), primarily androstenedione, testosterone and dehydroepiandrosterone (DHEA). During puberty, the adrenal gland contributes to androgen production in females (14). The adrenal gland also produces a unique set of 11-oxygenated 19-carbon steroids, which are emerging as clinic biomarkers of androgen excess (15), eventhough not currently used clinically in cases of androgen excess. It is not yet known how the 11-oxygenated 19-carbon steroids might contribute to premature adrenarche or accelerated bone age, however studies have demonstrated that these androgens are elevated in premature adrenarche, suggesting their putative role as bioactive androgens (16–18). One specific 11-oxyandrogen metabolite, 11-ketotestosterone (11KT) is notable as it displays similar androgenic activity to testosterone including activating the androgen receptor and is perhaps a clinically relevant potent agonist of the androgen receptor in humans (19).

Here, we report two patients with WSS due to deletion of *KMT2A*, one of which who presented with advanced bone age despite short stature and poor growth. This case report qualified for Institutional Review Board (IRB) exemption from the University of Alabama IRB.

Case report

Case 1: The patient is a 3 year and 11-month-old, African American girl diagnosed with WSS at nine months of age through a comparative genomic hybridization (CGH) array. Her weight is 13.7 kg (12th percentile), height is 97 cm (20th percentile), and the body mass index (BMI) is 14.5 kg/m^2 (26th percentile). She was a full term baby born *via* vaginal delivery with no known antenatal or perinatal complications. Her birth weight was 2.98 kg (6th percentile, z-score -1.50 SD) and birth length (0.2 percentile, Z score -2.76 SD) was 45.7 cm. Family history was notable for anemia in her brother and hypertension and diabetes mellitus in multiple relatives.

At 15 weeks of age, the patient was admitted to the hospital due to poor weight gain and failure to thrive (FTT). Hypotonia was noted, which was attributed to poor nutrition. Feeding therapy was initiated to address the apparent malnutrition. She was also found to have a moderate patent ductus arteriosus (PDA). The patient was discharged once feeding improved. The patient was admitted to the hospital at seven months of age for pneumonia refractory to outpatient treatment. During this stay, persistent feeding difficulties, lack of growth, developmental delay, and constipation were noticed. At nine months of age, the patient was found to have continued FTT with a weight of 5.44 kg (<1st percentile) and height of 64.50 cm (1st percentile). The PDA was closed at 10 months of age in a transcatheter procedure.

A CGH array was performed using the Agilent 4x180k aCGH + SNP array and revealed three DNA abnormalities in a peripheral blood specimen. The most relevant is 430kB deletion at 11q23.3 (117,965,223 and 118,394,787) (GRCh37/hg19), which encompasses 14 refSeq genes and includes *KMT2A*. Metaphase fluorescence *in situ* hybridization (FISH) analysis using the MLL (*KMT2A*) probe confirmed this deletion. Due to the inclusion of the *KMT2A* gene in this heterozygous loss-of-function pathogenic deletion, a diagnosis of WSS was made. This result includes the entirety of *KMT2A*, which is haploinsufficient, and therefore associated with a pathogenic loss-of-function, which is the mechanism of WSS. In addition, the microarray detected a variant of uncertain significance (VUS) 237kb deletion at 16p13.3 (6,943,310-7,180,460) [including *RBFOX1*, which may be associated with neurodevelopmental and neuropsychiatric disorders (19, 20)] and a 288kb duplication VUS at 2p16.3 (47,8909,992-48,179,514) which includes two OMIM genes – *MSH6* and *FBXO11*. Parental testing was not obtained due to cost.

The patient started occupational, physical, and speech therapy following her diagnosis of WSS, and a gastrostomy tube was placed at 22 months of age to address the continued feeding difficulties. At two years and 11 months of age, the patient's weight was 11.7 kg (8th percentile), height 87.1 cm (6th percentile), and BMI 15.4 kg/m^2 (37th percentile). The sex adjusted midparental target height was in the 90th percentile. A bone age evaluation was performed due to her short stature, which revealed advanced skeletal maturity more than two standard deviations above the mean using the Atlas of Greulich and Pyle – Figure 1. The carpal bones were noted to be closest to five years while the phalangeal bones were found to be between four years and 2 months and five years. Causes of advanced bone age such as hyperthyroidism, central precocious

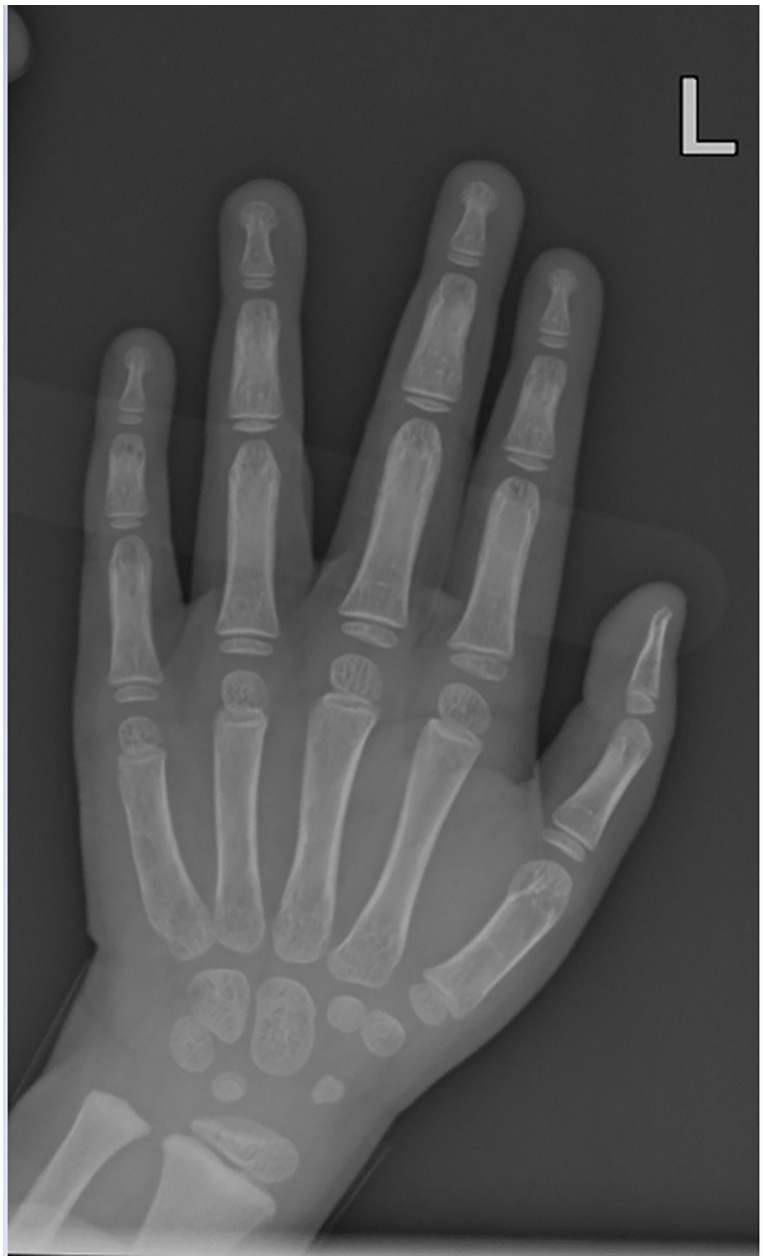

FIGURE 1
Bone age film for case 1, female child. The carpal bones were noted to be closest to five years while the phalangeal bones were found to be between four years and 2 months and five years using the Greulich and Pyle method, at chronological age two years and 11 months.

puberty, and congenital adrenal hyperplasia were excluded. At this time, there were no clinical signs of premature adrenarche or thelarche. Her growth velocity was noted to be normal at 8 cm/year. A repeat bone age was 6 years and 10 months, while the chronological age was 3 years and 11 months.

Evaluation for congenital adrenal hyperplasia (CAH) revealed normal results: baseline 17-hydroxyprogesterone 37 ng/dL, androstenedione 34 ng/dl, testosterone < 10 ng/dL, 11-deoxycortisol 53 ng/dL, DHEA 198 ng/dL, progesterone 0.1 ng/mL, cortisol 15.5 mcg/dL, deoxycorticosterone <16 ng/dL, and 17-hydroxypregnenolone 213 ng/dL. At the age of 3 years and 5 months, a serum 11-ketotestosterone (11KT) level was measured by commercial laboratory (Esoterix, Inc., Calabasas Hills, CA, USA) using liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). 11KT was 26.3 ng/dL, which is elevated when compared to published data on a

reference population of girls aged 4-5 years (range 7.3-10.9 ng/dL) and girls with premature adrenarche age 4-7 years (range 12.3-22.9 ng/dL) (17). The repeat 11KT at follow-up (chronological age 4 year 6 months) was still elevated at 33.8 ng/dL.

The patient continues to receive care at the same institution and has planned follow-up appointments.

Case 2: During the evaluation of Patient 1, a second child was identified at our institution with WSS due to an intragenic deletion in *KMT2A*. We reviewed his medical records as an early assessment of a patient with a possible similar risk profile to help to determine at what age the lab values or image abnormalities may begin to show differences. Case 2 is an 11-month-old male with dysphagia, hip dysplasia, decreased height for age (69 cm at 11 months with Z-score -3.2), and characteristic facial features of WSS. Genome sequencing revealed a *de novo* 5kb deletion of part of exon 27 and all of exon 28 in *KMT2A* (chr11:118503405-118508421 (GRCh38). Deletions are a rare mechanism of WSS. A baseline 11-ketotestosterone level was obtained (4.5 ng/dL). Bone age evaluation is not reliable in this patient as he is less than two years of age.

Discussion

Herein, we report the case of a patient with WSS with advanced bone age, concomitant with elevated 11KT, suggesting a possibility that 11-oxygenated C19 androgens could account for bone age advancement in some children with WSS. 11KT is postulated to be a dominant circulating bioactive androgen during normal and premature adrenarche (18). Following its secretion, 11KT can display androgenic effects by binding the androgen receptor (14). The 11-oxygenated steroids are primarily derived from adrenal tissue (16). These androgens, which have emerged as potential markers of adrenarche and conditions of androgen excess, are found to be particularly important in women and children (16). The potent androgenic 11-oxygenated steroids such as 11KT can bind and activate the human androgen receptor similar to testosterone and dihydrotestosterone (16). Due to this similar activity, it is therefore important to consider and measure these other androgens as they are clinically important especially in cases of androgen excess (20).

WSS syndrome affects males and females equally and is present among different populations with an overall estimated prevalence of <1 in 1,000,000 (21, 22). The genetic basis of WSS is a mutation in the *KMT2A* (also known as *MLL*) gene on chromosome 11q23, thought to result in haploinsufficiency of the gene and lead to the characteristic findings of the syndrome (3, 23). As *KMT2A* is expressed in a wide variety of tissues, WSS results in abnormalities in multiple body systems along with a wide variety of clinical findings (3). As the use of whole exome sequencing has increased, more individuals with WSS have been diagnosed, expanding the phenotypic spectrum of this condition. The molecular basis of most cases of WSS are single nucleotide variants (frameshifts, missense, nonsense, and splice-site variants) (10). Deletions are a rare mechanism which have only been reported once prior, in a female with advanced bone age and a deletion of exons 2-10 of *KMT2A* (12). This report adds an additional two cases of intragenic or whole-gene deletions of *KMT2A*, with one patient also having advanced bone age and a second patient whose evaluation did not reveal advanced bone age. However, this may be due to young age (11 months), so we will continue to monitor him for abnormal skeletal maturation.

Further research needs to be done to understand if elevated 11KT is seen in other patients with WSS, at what age exaggerated adrenal steroidogenesis is manifested, the potential reasons for this, and if there is an association between large multi-exonic deletions in *KMT2A* and advanced bone age. In case 1, the elevated 11KT marks an excess level of androgens that we hypothesize influenced bone maturation and led to the advanced bone age seen in this patient. Although our patient did not have significant clinical characteristics for premature adrenarche, her 11KT values were very similar (albeit slightly higher) to those reference intervals provided by the reference laboratory for premature adrenarche, while being 2.5 times higher than age-appropriate values.

The elevated 11KT level may indicate premature adrenarche due to the developmental maturation of the adrenal gland. The elevated 11KT in case 1 was not marked enough to suspect an adrenal tumor or warrant adrenal imaging. Moreover, other endocrinopathies, such as congenital adrenal hyperplasia, were excluded. The lack of clinical phenotype of premature adrenarche while having elevated urinary C_{19} steroids from age 3 years has been reported previously (24). We postulate that in some cases of WSS, this process of early adrenal maturation could be exponentially pronounced, accounting for elevated serum 11KT and accelerated bone age advancement.

Low birthweight and small for gestational age are known risk factors from metabolic programming for premature adrenarche (25). Early elevated 11KT may reflect the premature onset of adrenal gland maturation. The recent elucidation of conditions such as premature adrenarche and polycystic ovary syndrome manifesting with elevated 11KT (26), supports the notion of a mechanistic role for 11KT in premature adrenarche and conditions causing androgen excess. It is unclear if the increasing 11KT has a specific androgenic affinity for peripheral tissues such as bone and promotes skeletal maturation.

Genetic studies and mutational analysis indicate that the KMT family of histone lysine methylation-regulating enzymes interacts with the androgen nuclear receptor during hormone signaling (27). Interestingly, a genome-wide association study (GWAS) had shown that *KMT2A* can reportedly regulate steroidogenesis in animals (28). Perhaps the activity of the *KMT2A* gene might explain the abnormal steroidogenesis in WSS, accounting for the clinical phenotype of advanced bone age and ultimately short stature. Hence, it is possible that other

factors related to *KMT2A* may play a role in bone age maturation in some children with this genetic diagnosis. This case expands the association of WSS and advanced bone age and suggests an association between 11KT and advanced bone age in patients with WSS. To our knowledge, this is the first case that suggests a role of 11KT in bone age advancement in a patient with WSS. The patient's microdeletion does involve 14 total RefSeq genes, but the only one which has evidence for haploinsufficiency in the dominant state is *KMT2A*; others are not candidates for endocrinopathies (*MPZL2, UBE4A, CD3E, CD3G, CD3D, TMPRSS4, SCN4B, SCN2B, LOC100131526, ATP5MG, JAML, LOC101929089, MPZL3*), although this possibility cannot be excluded. Of note, the only prior reported patient with a deletion of *KMT2A* and advanced bone age had a deletion encompassing exons 2-10 (12). This more narrow deletion in a patient with a similar endocrinological phenotype gives some consistency that the *KMT2A* deletion is contributing to the advanced bone age and not another gene in our patient's microdeletion.

Our second patient with WSS due to *KMT2A* intragenic deletion had an unremarkable 11KT, but it is unclear if this was because he is still an infant at the time of testing, an age at which adrenal steroidogenesis may not have been stimulated. We thought the inclusion of this patient with WSS helped to underscore the possibility that adrenal steroidogenesis in patients with WSS might occur at different ages (similar to variations in adrenarchal age in normal children). We will continue to follow this as he ages. His bone age could not be accurately evaluated due to his young age. In a recent cohort of 104 patients with WSS (10), none had a multi-exonic or whole-gene deletion as in our patients, who both had a clinical diagnosis of WSS. Therefore, these patients provide additional evidence that multiexonic deletions are a mechanism for loss-of-function and the WSS phenotype, which had previously only been described in the report by Mendelsohn, B.A., et al. (12). Also, in Sheppard, S.E., et al. (10),, bone ages were normal in 55.2%, advanced in 24.1% and delayed in 13.8% of 29 patients with WSS. Further studies are needed to see if advanced bone age is a major feature of WSS and if there is a genotype-phenotype correlation with the mechanism of *KMT2A* variation and endocrinological characteristics.

We recognize that our novel observation was limited to positive findings in one patient, and hence, this observation may not be generalizable. While the elevated 11KT cannot be definitively considered as the cause for advanced bone age in this case, with what is known about premature adrenarche, the elevated 11KT levels could possibly be responsible for the skeletal maturation seen in this patient. Systematic longitudinal studies in children with WSS are warranted to address the apparent link between premature adrenarche, advanced bone age, and the pathophysiologic role of elevated 11KT, and to ensure reproducibility. We also acknowledge that the reference ranges for 11-oxygenated androgens in pediatric populations have been limited to two publications based on small sample sizes (17, 18), and further studies are needed to validate age and sex-specific reference intervals. We do not have a DHEAS measurement in Case 1, however the DHEA concentration of Case 1 was normal. We also do not have any information on the diurnal variation of the 11KT production and the estradiol concentrations.

Traditionally, aromatization of androgens to estrogens is postulated to contribute to the advancement of bone age in patients with precocious puberty. However, at this time, it is not known whether 11KT is aromatizable. Another possibility is that other androgens, specifically 11-oxygenated androgens, could be produced from the adrenal gland and may cause advanced bone age, which is not a known association at the time of this writing. Serum androgen concentrations may also not correlate completely with bone age advancement at the epiphyseal level. While this report serves as a hypothesis-generating concept, more patients with diagnosed WSS need to be studied to generalize this finding.

Conclusion

This case report highlights the potential role of elevated levels of 11KT in the clinical phenotype of elevated bone age observed in some patients with WSS. Further research needs to be done to elucidate the mechanism behind the elevation of 11KT in WSS and continue to expand the clinical phenotype of this syndrome. In addition, we present multi-exonic deletions as a mechanism of WSS; more studies are needed to determine if there is a genotype-phenotype correlation between large *KMT2A* deletions and bone age acceleration.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

KB performed outside research and literature review as well as patient chart review, authored first draft of manuscript, and edited subsequent drafts. EG and BS edited manuscript drafts. AH provided genetics perspective as well as edited drafts and contributed to writing. AA edited multiple drafts of manuscript, contributed to writing, helped with clinical context, and helped

oversee manuscript development. 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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Longitudinal evaluation of breast tissue in healthy infants: Prevalence and relation to reproductive hormones and growth factors

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Introduction: Breast tissue in infancy is a rather undescribed phenomenon. We aimed to describe the prevalence and progression of palpable breast tissue in healthy boys and girls aged 0-1 years and to evaluate clinical markers, individual serum hormone concentrations as well as combined hormone profiles as determinants of the persistence of breast tissue.

Methods: In total, 233 term infants (119 boys, 114 girls) were included and followed from birth until 1 year of age in The COPENHAGEN Minipuberty Study (ClinicalTrials.gov #NTC02784184). Infants were followed up to six times with a clinical examination and serum sampling. Principal component analyses (PCAs) produced combined hormone profiles.

Results: A total of 98% of all infants aged 0-1 year exhibited breast tissue at some point. 50% still had breast tissue present at 0.5-0.6 years in girls and 0.3-0.4 years in boys ('persistent'). At one year, more girls than boys had breast tissue present (p=0.010). Most clinical and hormonal markers did not differ in infants with/without persistent breast tissue. However, in those with persistent breast tissue, estradiol (first visit, girls, p=0.034), androstenedione, corticosterone, cortisol (first visit, boys, all p<0.050), length (first visit, boys, p=0.030), and testicular volume (0.3-0.4 years, p=0.040) were higher, while IGF-I (0.3-0.4, boys, p=0.033) was lower. In boys, a combined, PCA-derived hormone profile (first visit) was able to predict the persistence of breast tissue (area under the curve=83%) better than any single marker.

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Discussion: Palpable breast tissue in infancy is common in both sexes although it persists in significantly more girls than boys at one year of age. Data supports both the early origin of breast tissue (*in utero*- and early postnatal) as well as a role of endogenous hormone production in later development and maintenance.

KEYWORDS

thelarche, reproductive hormone, minipuberty, infancy, breast tissue, breast development, PCA, principal component analysis

Introduction

The appearance of palpable breast tissue is a common occurrence in both girls and boys. In pubertal girls, the attainment of palpable glandular breast tissue marks the onset of puberty, also known as thelarche. In pubertal boys, palpable glandular breast tissue is termed gynecomastia and is observed in approximately 50% of boys (1), with reports ranging from 20-70% (2–4). In infancy, the appearance of breast tissue has also been reported as a common finding with a prevalence of up to 90% in cross-sectional studies (3, 5, 6). However, literature on breast tissue in infancy is scarce. Breast tissue before the first year of life is usually considered harmless (7), but distinguishing between a normal and a pathological occurrence, i.e. precocious puberty, can in some cases be difficult (8, 9) and cases of infants referred to pediatricians due to breast development in infancy are not uncommon (10, 11). To enable the distinction between physiological and pathological breast development, further studies describing not only the prevalence of breast tissue *via* cross-sectional cohorts, but also the development and progression in a healthy cohort of infants followed longitudinally are needed.

Breast tissue and the underlying physiological causes in both pubertal girls and boys have been the focus of several studies. The causes of thelarche in girls, a fundamental pubertal milestone, have been suggested to include ovarian estradiol, but also the peripheral conversion of adrenal androgens to estrogens as well as body composition can cause growth of glandular breast tissue (12–16). Likewise, the cause of gynecomastia in pubertal boys has been suggested to be associated with estrogens, an altered estradiol/testosterone ratio, local sex steroid imbalances, luteinizing hormone (LH), growth hormone as well as insulin-like growth factor-I (IGF-I) (1, 17–21). Endocrine disrupting chemicals have also been suggested to play a role in both sexes, although this topic remains debated (22–25).

Studies of breast tissue in puberty have alluded to the underlying hormone profiles, yet similar studies concerning infancy are rare. Sex differences in the presence of breast tissue later in infancy (26) and breast tissue size have been reported (5, 27, 28) as well as correlations with serum and urinary estradiol in girls (5, 28) and umbilical cord testosterone in boys (27). As such, it still remains unknown whether breast tissue in infancy derives from and is maintained by placental hormone production *in utero*, endogenous hormone production in the infant, or both (26).

Principal component analysis (PCA) is a method of data simplification in which multiple variables are condensed into new variables, e.g. multiple hormones can be condensed into a single hormone profile. Such PCA-derived, combined hormone profiles have, for example, been shown to be better at detecting the presence of breast tissue in pubertal girls better than any single hormone (29).

The current study therefore aimed 1) to describe the prevalence and progression of palpable breast tissue in healthy boys and girls aged 0-1 years of age, and 2) to evaluate individual postnatal clinical markers and hormone concentrations as well as PCA-derived hormone profiles as determinants of the persistence of breast tissue in infancy.

Materials and methods

The COPENHAGEN minipuberty study

Healthy expectant mothers and their offspring were recruited as part of The COPENHAGEN Minipuberty Study hosted by the Department of Growth and Reproduction, Rigshospitalet, Copenhagen. A total of 233 full-term infants (119 boys and 114 girls), born term at gestational ages 38+0 to 41 +5, were included and followed from birth until 1 year of age. Infants were followed a maximum of six times including a clinical examination and serum sampling. A total of 186 infants completed the entire follow-up period. Further details on the design of the study have previously been described (30). All included infants were healthy and, importantly, not suspected of any endocrinological disorders.

In this current study, all 233 infants had information available on breast tissue at least at one visit. The infants were examined a total of 1,201 times, of which 1193 examinations included information on breast tissue (631 examinations of boys and 562 examinations of girls). These examinations took place between ages 4 days and 16 months of age. Details on clinical markers including body length, body weight, body mass index (BMI), feeding status, testicular volume (in boys), and breast tissue were included as well as serum hormone concentrations. No hormone stimulation tests were performed or medically indicated. Data from patient files on diagnoses given after the study follow-up was unfortunately not available.

Length was determined using a baby length measuring mat (ADE Germany GmbH & Co, Germany) to the nearest 0.5 cm, while weight was determined (without clothes or diapers) on an electronical scale (Baby-scale, Solotop Oy, Finland) to the nearest 0.005 kg. Feeding status was determined using questionnaires and at each visit an infant was grouped as either 1) breast fed only; 2) mixed breast milk, formula, and/or solids; or 3) formula only. Testicular volume was determined by ultrasonography (Hitachi Aloka SSD 500, Mechelen, Belgium) in terms of length and width (mm). An ellipsoid shape was assumed: volume = width x height^2 x $\pi/6$. The presence of breast tissue was determined by palpation (differentiated from pseudo-tissue due to adipose tissue). Both uni- and bilateral breast tissue was defined as the presence of breast tissue. Breast tissue size was measured to the nearest millimeter using a caliper. Breast tissue was deemed transient if it was present at one exam, absent at the next and then present again at a later exam.

Blood samples were acquired from a total of 211 infants (113 boys and 98 girls) comprising 641 samples (338 from boys and 303 from girls). Samples were drawn between five days and 14.2 months of age between 8 a.m. and 4 p.m.

Hormone assays

Due to limitations of the amount of blood that can be drawn from a healthy infant for research purposes (ethically as well as physically, refer to Busch et al. (30) for details), there was not a complete overlap in the hormones analyzed between the two sexes. In girls, LH, follicle-stimulating hormone (FSH), inhibin B, anti-Müllerian hormone (AMH), estrone (E1), estradiol (E2), sex hormone-binding globulin (SHBG), IGF-I, and insulin-like growth factor-binding protein 3 (IGFBP3) were quantified. In boys, LH, FSH, inhibin B, AMH, testosterone, androstenedione, cortisol, corticosterone, cortisol, 11-deoxycortisol, 17-hydroxyprogesterone, E1-S, SHBG, IGF-I, and IGFBP3 were quantified.

The following analytical methods were used: 1) LH and FSH: time-resolved fluoroimmunometric assays (AutoDELFIA, Perkin Elmer, Turku, Finland, Research Resource Identifier (RRID): For LH: AB_2783737, https://antibodyregistry.org/search.php?q=AB_2783737; for FSH: AB_2783738, https://antibodyregistry.org/search.php?q=AB_2783738) with limits of detection (LOD) of 0.05 IU/L and inter-assay coefficients of variation (CVs) ≤3% for both; 2) Inhibin B: double antibody enzyme-immunometric assay (Inhibin B GenII ELISA, Beckman Coulter, Brea, CA, USA, RRID : AB_2827405, https://antibodyregistry.org/search.php?q=AB_2827405) with an LOD of 3 pg/mL and a CV of <11%; 3) AMH: chemiluminescent immunoassay (Access 2 Immunoassay System, Beckman-Coulter, Brea, CA USA, RRID: AB_2892998, https://antibodyregistry.org/search?q=AB_2892998 for AMH)with an LOD of 0.14 pmol/L and a CV of <6%; 4) SHBG: chemiluminescent assay (Access 2 Immunoassay System, Beckman-Coulter, Brea, CA USA, RRID: AB_2893035, https://antibodyregistry.org/search?q=AB_2893035) with an LOD of 0.33 nmol/L and a CV of ≤10%; 5) estrogens and androgens: in-house liquid chromatography-tandem mass spectrometry (LC-MS/MS) (31, 32) with the following LODs and CVs for three times three different control samples per batch: E1 (LOD: 2.9 pmol/L, CV: 5-7%), E2 (4 pmol/L, 5-7%), testosterone (0.012 nmol/L, 2-6%), androstenedione (0.042 nmol/L, 4-8%), DHEAS (19 nmol/L, 4-10%), cortisol (1.9 nmol/L, 3-6%), corticosterone (0.1 nmol/L, 4-12%), cortisone (0.19 nmol/L, 5-7%), progesterone (0.036 nmol/L, 3-4%), 11-deoxycortisol (0.017 nmol/L, 3-13%), 17-hydroxyprogesterone (0.1 nmol/L, 3-7%), estrone 3-sulphate (0.026 nmol/L, 7-8%); and 6) IGF-I and IGFBP3: chemiluminescence assays (IDS-iSYS, Immuno-diagnostic Systems LTD, Bolton, United Kingdom) with LODs of 10 ng/mL and 80 ng/mL, respectively, and CVs of <8% for both.

For all hormones, concentrations below LOD were reported as LOD/2. The Danish Accreditation Fund (DANAK) for medical examination accredited all the above-mentioned analytical methods according to a European and International standard (the DS/EN ISO 15189).

Statistical methods

Firstly, Pearson's Chi-Squared and Fischer's exact tests were used to test for sex differences in the prevalence of breast tissue at the first and the last exams, respectively. Spearman's rho was used to examine correlations between continuous markers and size of the largest breast tissue (mm). P-values were considered significant at $p < 0.05$.

Secondly, part of the study aim was to investigate the underlying hormones associated with breast tissue in infancy. When reviewing the longitudinal data, it was apparent age intervals at 0.3 – 0.4 years (boys) and 0.5 – 0.6 years (girls) were associated with the disappearance of the breast tissue in approximately half of infants in each sex, respectively (still present in 30/62 boys and 31/64 girls). We therefore focused

our further analyses on this clinically evident dichotomy and consequently defined breast tissue as persistent if still present at these ages in boys and girls, respectively, and as non-persistent if it had disappeared.

To elucidate the biochemistry underlying the persistence of breast tissue in infancy, we therefore evaluated individual hormones and combined endocrine profiles at the first visit (median age 11 days, range 4-35 days; a reflection of the peri-/neonatal period) and at the ages of 0.3-0.4 and 0.5-0.6 years in boys and girls, respectively (a reflection of endogenous hormones postnatally). At these two timepoints, we 1) by use of Mann Whitney U tests, Pearson's Chi-squared or Fischer's Exact tests, identified if any single markers (single hormone concentrations, height, weight, and feeding status) were significantly associated with the persistence of an individual's breast tissue, and 2) by use of PCAs (described in detail below), examined combined endocrine profiles and tested whether these were able to distinguish between children with persistent vs. non-persistent breast tissue.

PCA is a method of data dimension reduction in which all the variables in a dataset are reduced into a smaller number of new combined 'variables' called principal components. By weighing the variance contributed by each of the variables, the principal components produced in a PC analysis account for decreasing amounts of the total dataset variance. To enable the PCAs to account for relevant variance concerning the presence of breast tissue (and not just general interindividual variance attributed to growth, minipuberty as a whole etc), receiver operating characteristic (ROC) curves were used to identify the hormones that were best able to distinguish between infants with persistent and without persistent breast tissue. All hormones with ROC-derived areas under the curve >60% were included in the PCAs. For boys, these hormones were AMH, androstenedione, corticosterone, cortisol, FSH, and IGFBP3, while in girls these hormones were AMH, E2, IGF-I, and inhibin B. In short, PCAs were used to further elucidate the biochemistry ('endocrinological profiles') of infants with and without persistent breast tissue and to investigate any differences in hormone concentrations between the two groups.

PCA were subsequently performed for each sex separately. Principal components with an Eigenvalue>1 ('Kaiser rule', Eigenvalue is an expression of the standard deviation of a dataset) were deemed viable, and corresponding principal component scores were calculated. These scores each represented a new, combined variable for each child. The abilities of the PC scores to predict the presence of persistent breast tissue were assessed using ROC. AUCs were applied to evaluate the performance of the principal component scores to distinguish between infants with persistent breast tissue from those without: ≥90%: excellent, ≥80-90%: good, ≥70-80%: fair, ≥60-70%: poor, and ≤60%: bad (33, 34). For the principal component scores with the best ability to do so for each sex, the endocrine profiles, i.e. the combination of hormones, underlying this principal component were then evaluated by their correlation coefficients in the given principal component. The correlations coefficients are an expression of the relative importance of the included hormones and values > ± 0.4 were deemed strong correlations (35).

In this study, no correction for multiple testing in the Mann-Whitney U tests was carried out. Dimension reduction by PCA inherently overcomes the problem of multiple testing as the different variables are condensed principal components.

Ethical considerations

The study has been registered with Clinical Trials.gov (#NTC 02784184). Parents consented in writing as well as orally to the participation of their child The COPENHAGEN Minipuberty Study. The study was approved by the regional Ethics Committees (H-15014876) and the Danish Data Protection Agency (RH-2015-210, I-Suite: 04146).

Results

Prevalence and progression of breast tissue in infancy

The presence of breast tissue was very frequent in both sexes (Figure 1). At the initial exam (boys' median age: 11 days, range: 4-36; girls: 11 days, 5-33) 114 of 116 boys (98%, three had their first examination at a later age) and 108 of 112 girls (96%, two had their first examination at a later stage) exhibited breast tissue. In total, three boys (3%) and two girls (2%) did not have palpable breast tissue at any examination during the first year of life. At the last exam (boys' age: 12.0 months, 11.2-15.9; girls: 12.0 months, 10.4-15.0), one of 75 boys (1%) and nine of 58 girls (16%) still had palpable breast tissue present, and a marked sex difference was therefore present at the last exam ($p = 0.010$) but not a the first ($p = 0.763$).

In 25 of 119 boys (21%) and in 22 of 114 girls (19%) transient breast tissue was observed (Figure 1). There was no observed difference between infants with and without transient breast tissue in terms of body weight, length, or BMI (all $p > 0.05$).

The median diameter of breast tissue at the peak diameter was 13 mm (IQR: 10-16) in boys and 13 mm (9–16) in girls. No apparent associations between concentrations of any of the analyzed hormones and breast tissue size were observed, all Spearman's rhos were < ± 0.2 (data not shown).

Determinants of the persistence of breast tissue in infancy

The majority of the individual markers measured did not display any significant differences in infants with persistent

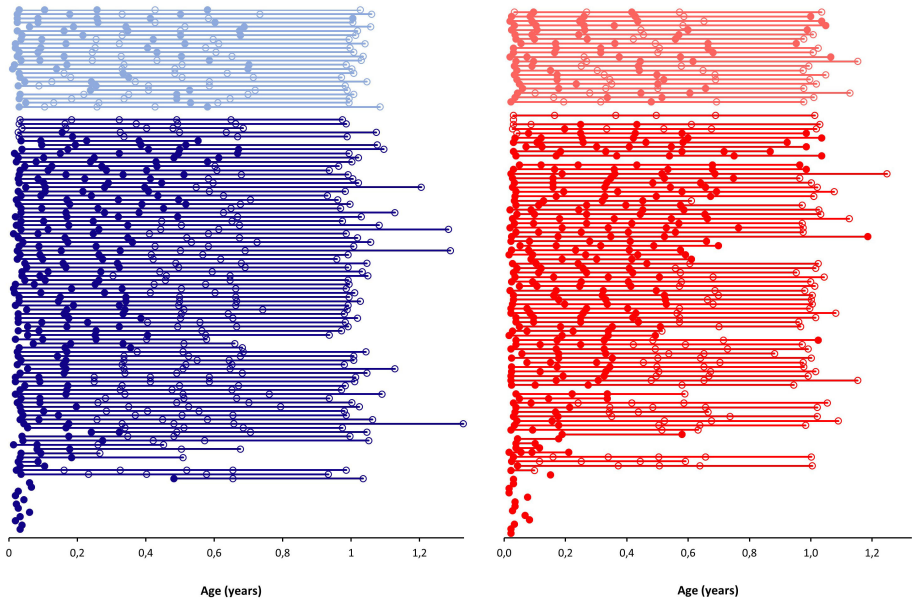

FIGURE 1
The prevalence of palpable breast tissue during the first year of life in boys (blue) and girls (red) according to age (years). Each connected line represents a single child. Circles indicate examinations; open circles are exams without the presence of palpable breast tissue, while closed circles are exams with the presence of palpable breast tissue. Lighter shades indicate children in which transient breast tissue was present, i.e. the appearance, disappearance and reappearance of breast tissue at consecutive examinations.

breast tissue vs. those with non-persistent breast tissue, i.e. palpable breast tissue at these respective ages in each sex (Tables 1, 2). However, in girls, estradiol concentrations measured at the first visit were significantly higher in those with persistent breast tissue ($p = 0.034$, Table 2 and Figure 2). In boys, serum concentrations of androstenedione, corticosterone, and cortisol measured at the first visit were all significantly higher in boys with non-persistent breast tissue (all $p < 0.05$, Table 1 and Figure 2). Serum concentrations of IGF-I at 0.3-0.4 years were also significantly higher in those with non-persistent breast tissue ($p = 0.033$, Table 1 and Figure 2). Conversely, boys with persistent breast tissue were significantly longer at the first examination than those without ($p = 0.03$, Table 1 and Figure 2). Furthermore, boys with persistent breast tissue had a larger testicular volume at 0.3-0.4 years ($p = 0.04$, Figure 2). Feeding status did not appear to be associated with the persistence of breast tissue (all $p > 0.05$).

Combined hormone profiles obtained by PCA are outlined in Tables 3, 4. In boys, the combined hormone profile at the first visit specified by principal component 2 was able to predict the persistence of an infant's breast tissue (AUC = 83%, i.e. 'good'). In girls, the best principal component at the first visit displayed fair abilities (AUC = 72%) to predict the persistence of the breast tissue based on the hormones from the first visit. PCA-derived hormone profiles at ages 0.3-0.4 or 0.5-0.6 years in each sex respectively displayed AUCs of 46 – 57%, i.e. 'bad'. However, the second principal component in boys at this age had a positive predictive value of 100%.

In boys, the correlation coefficients in the best PCA model (first visit), FSH, AMH, and androstenedione showed strong correlations to the profile (all $> \pm 0.4$). These correlations and the ability of the hormone profile to distinguish between breast tissue persistence are both similarly visible in the corresponding biplot, which visualizes the relative and separate clustering of those with and without persistent breast tissue (Figure 3). In girls, there were strong correlations ($> \pm 0.4$) for AMH, inhibin B and estradiol displayed in predominant (best) principal component, PC1 (first visit).

Discussion

In this cohort study of 233 healthy infant boys and girls followed longitudinally throughout the first year of life, palpable breast tissue was a very common finding. Shortly after birth there was no difference in the prevalence between the sexes, while at one year of age, breast tissue was still present in 16% of girls but only in 1% of boys. Differences in adrenal androgens, estradiol, body length, and principal component analysis-derived combined hormone profiles shortly after birth alluded to the breast tissue originating either *in-utero* or in early postnatal life, while differences in IGF-I and testicular volume

TABLE 1 Median hormone concentrations and anthropometric measures and total counts (n) in boys with persistent and non-persistent breast tissue in infancy at the initial exam (median age 11 days) and at the exam between 0.3-0.4 years of age.

BOYS	11 days				0.3-0.4 years			
	Non-persistent	Persistent	p-value	n	Non-persistent	Persistent	p-value	n
LH (IU/L)	4.5	5.2	0.298	31	1.3	1.4	0.782	28
FSH (IU/L)	1.6	2.1	0.091	31	0.7	0.7	0.337	29
Inhibin B (pg/mL)	206	190	0.535	31	368	287	0.114	22
AMH (pmol/L)	552	504	0.246	31	1603	1237	0.116	29
Androstenedione (nmol/L)	1.3	0.8	**0.029**	34	0.5	0.3	0.106	29
Corticosterone (nmol/L)	5.2	1.7	**0.006**	34	5.8	5.7	0.983	29
Cortisol (nmol/L)	129	45.5	**0.046**	34	226	148.	0.600	29
Cortisone (nmol/L)	94.0	106	0.903	34	58.5	45.9	0.163	29
E1-S (nmol/L)	0.3	0.01	0.213	34	0.01	0.01	0.301	29
DHEAS (nmol/L)	672	709	0.521	34	172	112	0.093	29
Progesterone (nmol/L)	0.5	0.3	0.238	34	0.1	0.04	0.078	29
Testosterone (nmol/L)	4.1	3.8	0.532	34	2.4	1.9	0.060	29
11-deoxycortisol (nmol/L)	1.3	1.3	0.849	34	2.3	1.0	0.359	29
17-hydroxyprogesterone (nmol/L)	2.3	2.1	0.591	34	1.4	0.7	0.198	29
SHBG (nmol/L)	102	89.7	0.561	23	154	169	0.828	25
IGF-I (ng/mL)	92	91	0.529	29	51	44	**0.033**	27
IGFBP3 (ng/mL)	1758	1951	0.164	29	2155	2095	0.099	27
Weight (g)	3607	3960	0.051	57	7246	7073	0.907	58
Length (cm)	53.0	54.7	**0.030**	57	66.0	66.2	0.462	58
Testicular volume (mL)	0.34	0.34	0.743	57	0.38	0.48	0.030	60

P-values express any differences observed between the two groups. Significant p-values (< 0.05) are highlighted in bold. Decimals have been rounded to nearest clinically meaningful number.
n, total count; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; E1-S, estrone sulphate; DHEAS, dehydroepiandrosterone-sulphate; SHBG, sex hormone-binding globulin; IGF-I, insulin-like growth factor-I; and IGFBP3, insulin-like growth factor-binding protein 3.

TABLE 2 Median hormone concentrations and anthropometric measures and total counts (n) in girls with persistent and non-persistent breast tissue in infancy at the initial exam (median age 11 days) and at the exam between 0.5-0.6 years of age.

GIRLS	11 days				0.5-0.6 years			
	Persistent	Non-persistent	p-value	n	Persistent	Non-persistent	p-value	n
LH (IU/L)	0.4	0.7	0.617	34	0.1	0.1	0.806	29
FSH (IU/L)	4.3	5.3	0.692	34	4.8	3.8	0.337	29
Inhibin B (pg/mL)	37	11	0.183	29	37	50	0.843	22
AMH (pmol/L)	2.9	4.6	0.212	30	10.5	29.3	0.565	28
Estrone (pmol/L)	14.4	25.1	0.539	29	4.9	4.6	0.356	28
Estradiol (pmol/L)	8.4	3.7	**0.034**	29	14.1	21.3	0.299	28
SHBG (nmol/L)	87.0	84.5	0.918	30	148.3	144.0	0.730	28
IGF-I (ng/mL)	88	88	0.069	28	46	36	0.734	27
IGFBP3 (ng/mL)	2206	2023	0.908	28	2238	1847	0.961	27
Weight (g)	3553	3330	0.583	62	7507	7768	0.248	64
Length (cm)	52.5	52.5	0.277	62	68.0	67.0	0.226	64

P-values express any differences observed between the two groups. Significant p-values (< 0.05) are highlighted in bold. Decimals have been rounded to nearest clinically meaningful number.
n, total count; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AMH, anti-Müllerian hormone; SHBG, sex hormone-binding globulin; IGF-I, insulin-like growth factor-I; and IGFBP3, insulin-like growth factor-binding protein 3.

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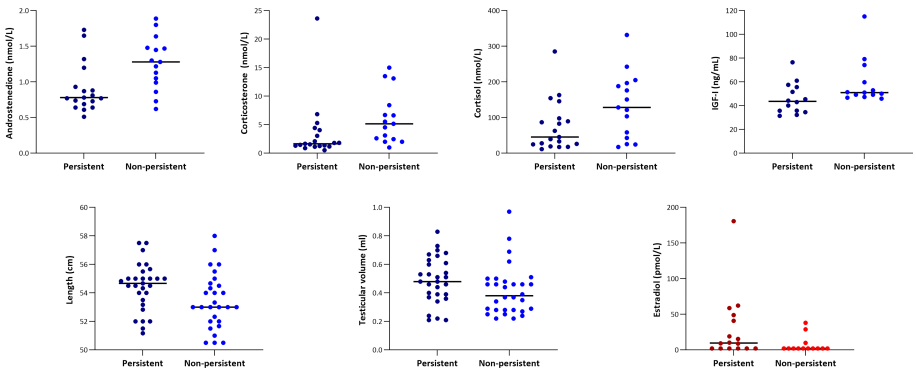

FIGURE 2
Variables with significant differences between infants with persistent (darker shades) and infants with non-persistent (lighter shades) breast tissue. For the boys (blues), there were significant differences between the groups at the first visit (median age 11 days) for androstenedione, corticosterone, cortisol, and length, while for IGF-I and testicular volume the difference was significant at the exam between 0.3-0.4 years of age. For the girls (reds), estradiol concentrations were significantly different between the groups at the first visit (median age 11 days). Breast tissue was labelled as 'persistent' if still present at 0.3-0.4 years of age in boys and at 0.5-0.6 years of age in girls.

later in infancy indicated that endogenous hormone production also plays a role in the maintenance of breast tissue in infancy.

In total, 98% of all infants had palpable breast tissue at some point in time during the first year of life, which is in line with or slightly higher than previous reports (3, 6, 36). At one year of age, there was a marked sex difference with the presence of breast tissue being more frequent in girls. However, transient breast tissue was a phenomenon present in a substantial proportion of both boys and girls. This has been scarcely described in infancy (37), but is a well-known occurrence in pubertal boys (gynecomastia) and girls (transient thelarche) (4, 13, 16, 38). These observations are important to note as they may aid clinicians in distinguishing between the normal physiological occurrence of breast tissue in infancy and premature thelarche

TABLE 3 Principal components and correlation coefficients for hormone concentrations in boys at the initial exam (median age: 11 days) and at the exam between 0.3-0.4 years of age.

BOYS	11 days		0.3-0.4 years	
	PC1	PC2	PC1	PC2
Correlations coefficients				
Corticosterone	-0.610	0.233	-0.537	0.231
Androstenedione	-0.169	0.445	-0.476	0.005
Cortisol	-0.643	0.164	-0.533	0.277
FSH	-0.122	-0.592	-0.190	-0.686
IGFBP3	-0.239	-0.384	0.334	-0.059
AMH	0.337	0.472	0.231	0.639
PCA-derived values				
Variance explained (%)	35	25	39	24
Cumulative variance (%)	35	60	39	63
Eigenvalue	2.11	1.47	2.33	1.47
ROC results				
AUC (%)	53	83	46	57
PPV (%)	50	73	64	100
NPV (%)	72	88	67	61
ACCURACY (%)	64	82	65	65

The ability of principal component-derived combined hormone scores to distinguish between boys with persistent and non-persistent breast tissue was evaluated by Receiver operating curves (ROC).
PC, principal component; FSH, follicle-stimulating hormone; IGFBP3, insulin-like growth factor-binding protein 3; AMH, anti-Müllerian hormone; PCA, principal component analysis; ROC, receiver operating characteristics; AUC, area under the curve; PPV, positive predictive value; and NPV, negative predictive value.

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TABLE 4 Principal components and correlation coefficients for hormone concentrations in girls at the initial exam (median age: 11 days) and at the exam between 0.5-0.6 years of age.

GIRLS	11 days		0.5-0.6 years	
	PC1	PC2	PC1	PC2
Correlations coefficients				
Estradiol	0.517	-0.222	0.516	0.102
IGF-I	0.170	0.962	-0.175	0.949
Inhibin B	0.588	0.067	0.697	-0.035
AMH	0.598	-0.147	0.467	0.296
PCA-derived values				
Variance explained (%)	56	25	46	25
Cumulative variance (%)	56	81	46	71
Eigenvalue	2.22	0.99	1.85	1.02
ROC results				
AUC (%)	72	63	51	57
PPV (%)	75	71	63	67
NPV (%)	77	61	67	60
ACCURACY (%)	76	64	64	64

The ability of principal component analysis (PCA)-derived combined hormone scores to distinguish between girls with persistent and non-persistent breast tissue was evaluated by Receiver operating curves (ROC).
PC, principal component; IGF-1I insulin-like growth factor-I; AMH, anti-Müllerian hormone; PCA, principal component analysis; ROC, receiver operating characteristics; AUC, area under the curve; PPV, positive predictive value; and NPV, negative predictive value.

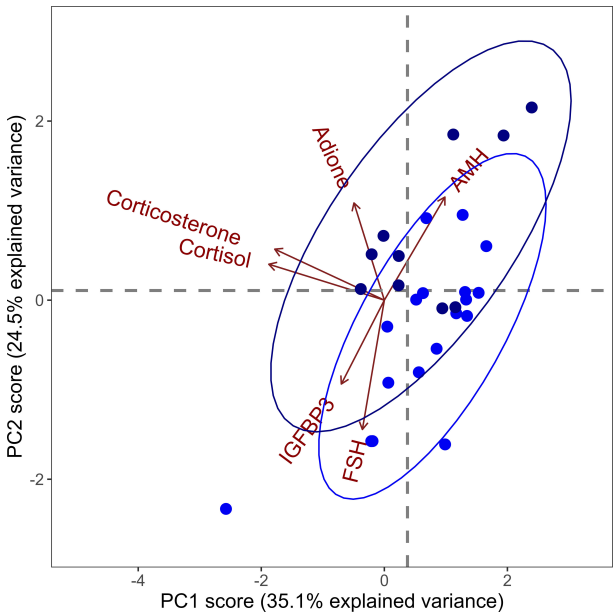

FIGURE 3
Principal component (PC) biplot for PC1 and PC2 based on hormone concentrations of anti-Müllerian hormone (AMH), adione (androstenedione), corticosterone, cortisol, insulin-like growth factor-binding protein-3 (IGFBP3) and follicle-stimulating hormone (FSH) in boys at the first visit (median age 11 days). These two principal components account for a total of 59.6% of the dataset variance. Arrows (i.e. vectors) represent the correlation coefficients of the hormones with the principal components, and these should be interpreted horizontally for PC1 and vertically for PC2. While cortisol and corticosterone were almost aligned in the horizontal plane, indicating a strong correlation with PC1, adione was almost aligned vertically, indicating a strong correlation with PC2. Darker blue dots represent boys with persistent breast tissue, while lighter blue dots represent boys with non-persistent.

(8, 9, 11, 39). Thus, in both boys and girls with breast tissue (transient or not) present during the first year of life as the sole sign of puberty, precocious puberty seems highly unlikely. Aside from the clinical application of our findings, breast tissue has also been suggested as a non-invasive means of monitoring exogenous estrogenization effects from environmental chemicals (40). Yet given the high prevalence of palpable breast tissue, as well as the transient nature in both sexes, breast tissue in early infancy as a proxy of chemical exposure would have inherent limitations. It is important to note that the high prevalence of breast tissue in both sexes in this study may reflect different aspects, including but not limited to: the method of palpation vs. ultrasound; demographic-specific results that may arise due to e.g. differences in feeding patterns (high degree of breast-fed v bottle-fed (41) and/or differences in exposure to endocrine disrupting chemicals (42, 43) both across and within countries. Moreover, it is important to note that while the current study elucidates that the presence of breast tissue, in both sexes, is a normal, physiological phenomenon in infants younger than 1 year of age, it does not investigate whether persistent breast tissue in infancy is a risk marker of future precocious puberty.

Altogether, the analyses concerning the underlying etiology of breast tissue development in infancy alluded to both *in utero*/early postnatal factors and later endogenous hormone production in the infant. Specifically, adrenal androgens and length in boys and estradiol concentrations in girls in early postnatal life indicated that *in utero* milieu and/or very early postnatal hormone production play a role in the initial development of breast tissue. Moreover, the ability of the PCA-derived, combined hormone profiles shortly after birth (first visit) to distinguish between infants with and without persistent breast tissue was further indication of the pre- and/or early post-natal origin. The correlation coefficients in the PCA analyses confirmed the role of androstenedione as well as FSH and AMH in regulating infant breast tissue boys. In girls, AMH, inhibin B, and estradiol were also strongly correlated at this age. Altogether, the data supports previous studies reporting that most infants were born with palpable breast tissue (26, 27, 44), which makes the fetal origin, at least in part, likely. In line with this, maternal estrogens have been mentioned in multiple studies as reasons for breast tissue development in infancy (40, 45). However, our study design, which lacks a perinatal examination, does not allow for an absolute distinction between the pre- and postnatal periods.

While our study may allude to a pre-/early postnatal origin of breast tissue in infants, the mere fact that some infants had breast tissue present several months after birth while others did not, as well as the transient phenomenon in some infants, indicates that endogenous hormone production plays a role in the maintenance of breast tissue as well (26). This has also been observed in other studies in which estradiol has been found to be associated with breast tissue diameter and development (5, 28). Estradiol production in girls has also been suggested as the reason for the marked sex difference in the latter part of infancy (28), a sex difference which was also observed in our study. This is likely ovarian estradiol with the known activation of the hypothalamic-pituitary-gonadal axis during minipuberty, although adrenal origin as well as peripheral aromatase action in the fatty tissue also cannot be ruled out.

The ability of testicular volume at 0.3-0.4 years of age to predict breast tissue persistence in our data also heavily suggests that the testicular hormone production is, at least in part, responsible for the maintenance of breast tissue in infant boys. Moreover, IGF-I at age 0.3-0.4 years was also found to be significantly lower in infant boys with persistent breast tissue. However, this is in contrast to reports of higher concentrations of IGF-I in boys with pubertal gynecomastia (1). While the overall performances of the combined hormone profiles produced by PCA later in infancy were not statistically meaningful, the PCA model summarizing newborn endocrine profiles in boys exhibited a positive predictive value of 100%, implying that endocrine profile scoring in this way with great certainty can predict the persistence of infant breast tissue. Although the ROC performances overall were poor, there were still indications that the combined hormone profiles later in infancy play a role in the persistence of breast tissue.

We applied PCA methodology to elucidate the discriminatory capabilities of combined hormone profiles on the persistence of infant breast tissue, and found that combined hormone profiles, primarily at the first visit but also later in infancy, were closely associated with persistence. Notably, in this study, PCA was used to investigate normal physiology rather than for a proposed direct clinical application. The PCA method is routinely used to assess dichotomic clustering in dataset variance; however, its application in endocrinological research is rather novel. The major advantage is its ability to condense multiple variables into a single principal component ('variable') that can allude to the underlying biochemistry of the phenotype in question. Summarizing the contributions of all included feature variables as one or two principal components eliminates the concerns of multiple testing and p-value overestimation. Although the hormone profiles investigated by PCA in this current study may not provide immediate clinical utility, characterizing pediatric development trajectories is an important aspect of pediatric endocrinology.

Aside from the novel use of PCA, the strengths of this study included: 1) the design of the cohort of healthy infants followed longitudinally allowing for the description of both prevalence and the progression of breast tissue in infancy; 2) the rather large study size of 233 infants; 3) the frequent serum sampling; and 4) the use of highly sensitive hormone analytical methods. However, the study also had limitations which included: 1) due to limitations in serum sample volumes, estrogens were not quantified in boys and androgens were not quantified in girls; 2) all infants were Caucasian which restricts generalizability; 3) the measurement of the size of the breast

tissue can be difficult and interobserver variation has previously been noted in both the current cohort (30) as well as other cohorts (5). This interobserver variation, which may also have been affected by infant weight/body size, could possibly have been limited by the use of breast ultrasound, which reportedly has a small intra- and interobserver variation (46); 4) infants contributed with multiple observations in the Spearman's rho analyses, yet the distribution of the multiple samples was random and there was no reason to believe that those who included more observations were outliers/at ends of the given scales; 5) the cohort was recruited in affluent areas of Copenhagen and consequently very few infants were formula-fed vs. breast milk-fed, which may have hidden true differences between the groups; and 6) *post-hoc* correction for multiple testing was not performed as it would have obscured the significant associations presented in the study. The study was exploratory in nature, and, as such, the weight attributed to a single significant value was limited. Additionally, only associations with a biological foundation and/or previously described by other groups were examined.

In conclusion, palpable breast tissue in infancy is very common in both sexes although it persists in significantly more girls than boys at one year of age. As in puberty, transient breast tissue (i.e. the appearance, disappearance, and reappearance) was very common in both sexes. The data presented on a whole supports both the early origin of breast tissue (*in utero*- and early postnatal) as well as a role of endogenous hormone production in later development and maintenance. Altogether, the presence of palpable breast tissue throughout the first year of life is a normal phenomenon in both sexes and may not alone warrant further endocrinological workup, although individual evaluation and management is important.

Data availability statement

The datasets presented in this article are not readily available because Danish/EU data protection legislation as well as ethical considerations do not allow for it. Requests to access the datasets should be directed to MLL, marie.lindhardt.ljubicic@regionh.dk.

Ethics statement

The studies involving human participants were reviewed and approved by The Regional Ethics Committees (H-15014876). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

ML, EU, MF, and AB all took part in study design, data collection, data processing, interpretation of data, and writing of the manuscript. HF, TJ, AJ, and CH took part in study design, data processing, interpretation of data, and writing of the manuscript. AM took part in data processing, interpretation of data, and writing of the manuscript. Lastly, ML and AM carried out all statistical analyses. 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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Diagnosis of childhood and adolescent growth hormone deficiency using transcriptomic data

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Background: Gene expression (GE) data have shown promise as a novel tool to aid in the diagnosis of childhood growth hormone deficiency (GHD) when comparing GHD children to normal children. The aim of this study was to assess the utility of GE data in the diagnosis of GHD in childhood and adolescence using non-GHD short stature children as a control group.

Methods: GE data was obtained from patients undergoing growth hormone stimulation testing. Data were taken for the 271 genes whose expression was utilized in our previous study. The synthetic minority oversampling technique was used to balance the dataset and a random forest algorithm applied to predict GHD status.

Results: 24 patients were recruited to the study and eight subsequently diagnosed with GHD. There were no significant differences in gender, age, auxology (height SDS, weight SDS, BMI SDS) or biochemistry (IGF-I SDS, IGFBP-3 SDS) between the GHD and non-GHD subjects. A random forest algorithm gave an AUC of 0.97 (95% CI 0.93 – 1.0) for the diagnosis of GHD.

Conclusion: This study demonstrates highly accurate diagnosis of childhood GHD using a combination of GE data and random forest analysis.

KEYWORDS

growth hormone deficiency, transcriptome (RNA-seq), machine learning, growth hormone, random forest - ensemble classifier

Introduction

Growth hormone deficiency (GHD) is a rare but important cause of short stature with a prevalence of approximately 1 in 4000 (1). Consensus guidelines recommend an approach integrating auxological, biochemical and radiological data for the diagnosis of GHD in childhood and adolescence (2). Pharmacological stimulation tests, in which a cut-off level is

selected for peak growth hormone (GH) levels below which the child is diagnosed with GHD, remain key to the diagnosis of GHD despite many known problems with these tests. These problems include poor reproducibility (3) and that the peak GH level achieved is affected by the pharmacological stimulus used (4), the GH assay (5) and body composition (6, 7). In one survey the peak GH level utilized for diagnosis varied between 6 and 10 μg/L in nine European and US national guidelines (8).

In addition to these problems most of the pharmacological stimulation tests require fasting and all require multiple blood samples. Both of these requirements can be challenging for small children, particularly those with significant medical problems e.g. history of extreme prematurity where venous access may be very challenging, in children with needle phobia or autistic spectrum disorders. Adverse effects such as vomiting and nausea are common and rarely serious adverse events such as cerebral edema have been associated with these tests (9). We have therefore sought to develop a gene expression-based test as a potential replacement for pharmacological stimulation tests. This would require only a single blood sample and, as no pharmacological stimulant would be needed, would avoid any significant adverse effects.

Gene expression based analysis has been utilized in the diagnosis of interstitial lung disease (10), kidney disease (11), atrial fibrillation (12), autism (13) and in the prognosis and classification of tumors (14–16). For both autism and atrial fibrillation peripheral blood gene expression was used for diagnosis (12, 13). We therefore hypothesized that gene expression signatures in peripheral blood could be a diagnostic tool for GHD with the potential to replace pharmacological stimulation tests. In an initial study (17) we compared gene expression profiles between 98 children with GHD enrolled in the PREDICT study (18) and 26 healthy control children whose gene expression data were obtained from online datasets. After selecting the 271 probesets whose expression correlated with peak GH levels a Random Forest classifier gave an Area under the Receiver Operating Characteristic Curve (AUC-ROC) of 0.95 (sensitivity 96%, specificity 100%) for predicting the diagnosis of GH indicating this had the potential to be an excellent diagnostic test (17). There were, however, several limitations to that initial study: 1) the control subjects used were healthy rather than short stature controls 2) the patient and control children were assembled from different studies and 3) GH stimulation test and assay were not standardized in the PREDICT study.

The aim of this study was to assess the utility of gene expression data for the diagnosis of GHD in a prospectively recruited cohort of children and adolescents undergoing pharmacological stimulation tests at a single tertiary pediatric endocrinology center.

Methods

Ethics and patients

This study was approved by the Bradford Leeds Research Ethics Committee (Reference 18/YH/0226 IRAS ID 231325) and conducted in accordance with Good Clinical Practice and the Declaration of Helsinki. Informed consent was obtained either from parents or from the young person themselves where they were over 16 and had capacity to give consent.

Patients were recruited from clinics of the Paediatric Endocrinology Department at the Royal Manchester Children's Hospital. Every patient attending the outpatient medical investigation unit for either an arginine or glucagon stimulation test was invited to take part in the study between 1st November 2018 and 31st January 2019. 44 patients were invited to take part and 27 (59%) both attended for their appointment and agreed to take part in the study. For three patients a result for the pharmacological stimulation test was not available due to difficulties with obtaining venous blood leaving 24 patients in the study. Auxological, biochemical and radiological data were obtained from the patients records. SDS scores for auxological data were calculated with Auxology 1.0 (Pfizer, New York, USA) using UK 1990 Cole Reference data.

Stimulation test protocols, sex steroid priming and biochemical assays

Arginine and glucagon tests were the only GH stimulation tests used in our institution during the study period. Protocols for the arginine and glucagon stimulation tests used in our institution are available at https://mft.nhs.uk/app/uploads/2022/03/Paediatric-DFT-Protocols-V7_Feb-2022.pdf (accessed 22nd August 2022). Sex steroid priming is given in our unit for pre-pubertal girls >8 years or boys >9 years undergoing stimulation tests. Ethinylestradiol 10-20 micrograms is given once daily for 3 days prior to the test for both boys and girls. A normal test result in our institution is indicated by a peak GH level ≥ 7 μg/L. GH, IGF-I and IGFBP-3 were measured on the IDS iSYS assay (Immunodiagnostic systems, Tyne and Wear, UK). The GH assay used is standardized to the recombinant GH calibration standard World Health Organization 98/574 and complies with recommendations on assay standardization (19).

Statistics, regression and random forest analysis

Differences in demographic characteristics were assessed *via* a Mann Whitney U test or Fisher's Exact test. A Random Forest algorithm (20) was used to predict GHD status. The data were unbalanced (8 GHD subjects and 16 controls) and with an unbalanced dataset Random Forest poorly predicts the minority class (in this case GHD subjects). To overcome this problem a synthetic minority over-sampling technique (21) was used to rebalance the dataset prior to Random Forest prediction using age, gender and transcriptomic data. The predictions were assessed based on the AUC-ROC and the out-of-box ROC curve (OOB-ROC) as a validation set. Identifying those probesets most likely to contain predictive capacity was achieved with the use of Boruta (22). All statistical analyses were performed using R 4.0.0.

Random Forest analysis does not require separate test and validation data sets as the OOB-ROC functions as a validation data set. In developing the random forest algorithm hundreds or thousands of decision trees (in our case 1000 trees) are created. Each tree is generated using a random selection of input variables and randomly selected two thirds of the subjects. Each tree produced a classification vote and the majority vote across all trees determined

final classification. For each tree there was a random one third of subjects whose data was not used in generating that tree – these data were then used to generate the OOB-ROC which essentially functions as a validation data set.

Boruta is used to define which of the input variables for the Random Forest are contributing to the predictive power. It does this by permuting the data (randomly shuffling the variables – in this case gene expression levels) to break any link between the input variables and outcome measured. These permuted variables are referred to as "shadow" variables and Boruta then runs a Random Forest algorithm using the shadow variables (with the same outcome measure as the original dataset) to define the range of predictive power of these shadow variables. The predictive power of the variables in the original data are then compared to the range of predictive power shown by the shadow variables. On the basis of that comparison the original variables (in this case individual gene expression levels) are classified as confirmed, tentative or rejected.

Gene expression analysis

Peripheral blood samples (2.5 ml) were taken into PAXgene tubes (Qiagen, Manchester, UK) and stored at -80°C for the separation of total RNA as a single batch. Total RNA was submitted to the Genomic Technologies Core Facility. Quality and integrity of the RNA samples were assessed using a 4200 TapeStation (Agilent Technologies) and then libraries generated using the TruSeq® Stranded mRNA assay (Illumina, Inc.) according to the manufacturer's protocol. Briefly, total RNA (1ug) was used as input material from which polyadenylated mRNA was purified using poly-T, oligo-attached, magnetic beads. The mRNA was then fragmented using divalent cations under elevated temperature and then reverse transcribed into first strand cDNA using random primers. Second strand cDNA was then synthesized using DNA Polymerase I and RNase H. Following a single 'A' base addition, adapters were ligated to the cDNA fragments, and the products then purified and enriched by PCR to create the final cDNA library. Adapter indices were used to multiplex libraries, which were pooled prior to cluster generation using a cBot instrument. The loaded flow-cell was then paired-end sequenced (76 + 76 cycles, plus indices) on an Illumina HiSeq4000 instrument. Finally, the output data was demultiplexed (allowing one mismatch) and BCL-to-Fastq conversion performed using Illumina's bcl2fastq software, version 2.17.1.14. BAM files were used to generate raw counts were mapped to the human reference genome (GCA_000001405.15 GRCh38 from NCBI).

The EdgeR package for R 4.0.0 (23, 24) was used to assess gene expression. Genes were filtered to remove low expression features. Across all cells, genes with fewer than 15 total counts were removed; the minimum count per cell for a feature to be considered expressed is 10. The large group size was set at 10 samples and genes had to be expressed in 70% of cells of smaller groups to pass filtering. Gene level normalization was performed using TMM (trimmed mean of M values). A block design in EdgeR was used to control for the impact of confounding factors (age and gender).

Gene ontology was performed using biological process data in the WebGestalt online toolkit (25). Clusters of related ontologies were defined and primary pathways identified using weighted set cover (26).

Sequence data is available *via* NCBI Gene Expression Omnibus as GSE190502. Individual patient data (age, gender, type of GH simulation test, peak GH level and use of sex steroid priming) is included in GSE190502 and as Supplementary Table 1.

Results

Patients

Of the 24 patients who were enrolled in the study and completed the diagnostic test eight were diagnosed with GHD and 16 did not have GHD. Of the eight patients with GHD five were diagnosed with GHD with a single test due to the presence of MRI abnormalities of the hypothalamo-pituitary axis. Two patients had isolated anterior pituitary hypoplasia, one anterior pituitary hypoplasia with an arachnoid cyst, one anterior pituitary hypoplasia combined with a thin stalk and multiple sclerosis and one patient had a small anterior pituitary with bulky optic nerves (this patient has a diagnosis of neurofibromatosis type 1). Two patients had a normal pituitary exam and were diagnosed with GHD on the basis of two independent GH stimulation tests. One patient was born small for gestational age, had a normal MRI pituitary and a CHARGE syndrome phenotype and was started on GH after a single test. Three patients in the non-GHD group had genetic disorders – one with Prader-Willi syndrome, one with Wiedemann-Steiner syndrome, one with a chromosome 21q deletion and one patient had a peroxisomal disorder. In the GHD group one patient had a genetic condition – neurofibromatosis type 1.

Six patients had glucagon stimulation tests and 18 arginine stimulation tests. All patients undergoing glucagon stimulation testing had a previous failed arginine stimulation test. The test was primed with sex steroids in seven cases. There were 13 male and 11 female patients. 18 patients were prepubertal, 2 patients had just started puberty (one girl at breast stage 2 and one boy with testicular volumes of 5 mL bilaterally) and 4 patients had either completed puberty or were near the end of puberty. Baseline clinical data (auxology and biochemistry) are given in Table 1. There was no significant difference between the GHD and non-GHD group for age, gender, birth weight SDS, height SDS, parental height adjusted height SDS, growth velocity SDS, weight SDS, BMI SDS, bone age delay, IGF-I SDS or IGFBP-3 SDS.

Our study included 3 patients who were evaluated as part of an end of growth assessment (having previously been treated with recombinant human GH for GHD). These subjects were not GHD with peak GH levels of 9.1 µg/L, 9.5 µg/L and 31 µg/L to arginine stimulation testing. End of growth patients previously treated with recombinant human GH were not included in the analysis of growth velocity SDS and or height SDS. The three patients evaluated as part of an end of growth assessment had stopped treatment with recombinant human growth hormone a minimum of six weeks before the end of the test, all had a low likelihood of continuing GHD and were thus retested as per recommendations in international guidelines (27). All other patients had not received recombinant human growth hormone treatment at the time of the study.

TABLE 1 Baseline characteristics of study participants.

	GHD (n=8)	GH sufficient patients (n=16)	P-value
Age (years)	8.6 (9.0)	9.0 (5.8)	0.52
Male Gender (n, %)	6 (75)	7 (43)	0.21
Birth Weight SDS	-0.7 (0.7)	-1.1 (1.7)	0.51
Height SDS	-3.0 (1.0)	-2.5 (0.7)	0.48
Weight SDS	-2.5 (2.4)	-2.1 (1.5)	0.98
Body Mass Index SDS	0.3 (1.7)	-0.1 (1.4)	0.32
Parental Adjusted Height SDS	-2.1 (0.3)	-2.5 (1.7)	0.70
Growth Velocity SDS	-1.5 (1.7)	-1 (3.3)	0.52
Prepubertal (N, %)	7 (87)	11 (68)	0.62
Bone Age Delay (years)	1.5 (1)	1.7 (1.4)	1.0
IGF-I SDS	-2.2 (1.7)	-1.5 (1.5)	0.20
IGFBP-3 SDS	-1.4 (1.6)	-0.8 (1.9)	0.33

Data is given as median (interquartile range) unless otherwise stated. Patients who had reached end of growth are not included in the analysis of growth velocity or analysis of height SDS.

Gene expression data

Of the 271 genes previously identified as predictive of GHD in relation to normal controls (17) 208 could be annotated to an ensemble external gene name and 160/208 passed through low expression filtering in the dataset of GHD patients (n=8) and short stature controls (n=16). A strict threshold to define expression was used in our new dataset hence the reduction in number of genes expressed in both datasets. Transcriptomic data was Log_2 normalized (TMM) and age and gender were treated as confounding factors in the data. Seven out of the 160 genes in the predictive group had significant differential expression (0.02<p<0.05 - *NOTCH3*, *LAYN*, *SHF*, *GRB10*, *SH3GLB2*, *CYB5A* and *SGSM2*).

A random forest algorithm after adjusting for imbalanced numbers using SMOTE (final data used 44 subjects: 20 short stature controls and 24 GHD patients) in this cohort gave an AUC of 0.95 (95% CI 0.89 – 1.00) for the diagnosis of GHD. Boruta (using 99 iterations) was able to identify 100/160 genes with predictive capacity greater than permuted data within the dataset (60 confirmed and 40 tentative, see Figure 1 and Supplementary Table 2).

Gene ontology of the genes with predictive ability identified a range of associated biological processes. The top fifty biological processes clustered by weighted set cover to 10 clusters of ontologies (see Figure 2) 0.0037<p<0.018. Pathways identified included regulation of TORC1 signaling and inositol phosphate metabolic processes.

We had previously identified the 10 genes with greatest predictive value in classification of GHD in relation to normal controls when combined with genetic data (17). Of these two were validated as being of predictive value in classification of GHD in comparison to short stature controls (*NRXN1 & PTGDS*).

Discussion

Our previous study (17) highlighted the potential for GE data to be used for the diagnosis of childhood GHD with a very promising AUC for predicting GHD status of 0.95 (95% CI 0.91 – 0.99) and our current study confirms that potential with an AUC of 0.97 (95% CI 0.93 – 1.0). We have changed from using Affymetrix HU 133 plus 2.0 arrays to an RNA sequencing based approach to measure GE thus moving from measurement of relative to absolute concentrations of RNA. This change in technique did mean that we could not use exactly the same random forest algorithm in this study as was generated in our original study, however, we did utilize the same

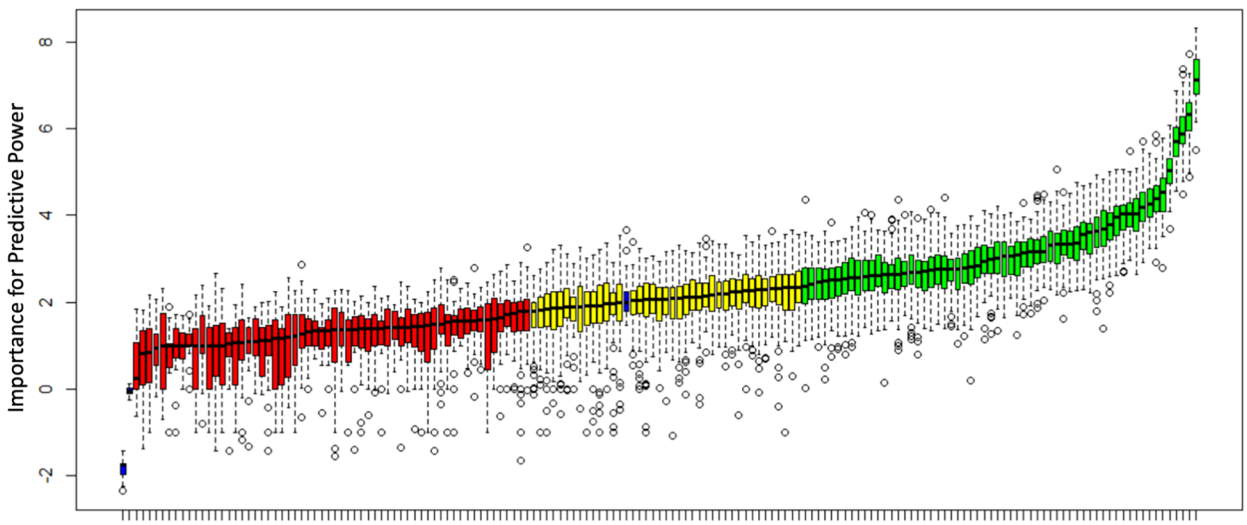

FIGURE 1
Analysis of predictive power for the diagnosis of GHD using Boruta. All 160 genes utilized in this study are shown and predictive power compared to shadow variables (blue bars) generated from permuted data. Genes are classified as confirmed, tentative or rejected based on a comparison with the shadow variables. 60 genes were confirmed (green), 40 tentative (yellow) and 60 rejected (red). A median importance score ≥ 2.359 resulted in a confirmed status, a score ≥1.785 and < 2.359 resulted in tentative status and a score <1.785 rejected status.

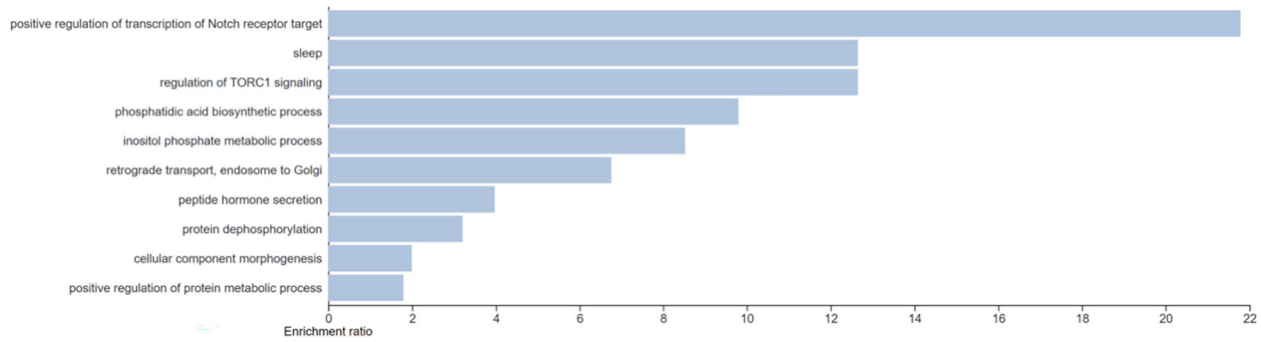

FIGURE 2
Gene ontology analysis of the genes with predictive capacity with clustering of the top 50 biological pathways *via* weighted set cover. 10 clusters of gene ontology were identified ($0.0037 < p < 0.018$).

set of 271 genes whose expression was related to peak GH levels in the original study. Of the 271 genes utilized in our original study 160 were present in the RNAseq data. The reduction in number is likely to reflect the strict cut-off used for filtering low expression genes in this study. We believe an RNA sequencing based approach is likely to achieve more widespread uptake in the future as many labs already use next generation sequencing approaches for DNA studies. The AUC for the gene expression based test is better than the current baseline tests used in the diagnosis of GHD namely IGF-I with an AUC of 0.73 and IGFBP-3 with an AUC of 0.8 (28).

The major limitation of our previous study was that we compared GE data from children with GHD to healthy controls (likely to be of normal stature) accumulated from several different datasets. In this study the control cohort was short non-GHD children who had undergone GH stimulation testing. One significant strength of the study is that we recruited an unselected real-world sample of patients undergoing GH stimulation tests who are likely to be generally representative of the patients that pediatric endocrinologists select for testing. There were no significant differences between the short non-GHD and GHD children for age, gender, height SDS, parental adjusted height SDS, growth velocity SDS or BMI SDS. The absence of any significant difference in height or parental adjusted height SDS between the GHD and non-GHD short stature children may be due to the low numbers of GHD subjects. IGF-I and IGFBP-3 SDS concentrations were lower in the GHD group but this was not significant. In children with GHD IGF-I and IGFBP-3 concentrations can be in the low or low normal range with suggested cut-offs for the diagnosis of childhood GHD of -1.6 SD for IGF-I and -1.8 for IGFBP-3 (29) whilst in children with idiopathic short stature low IGF-I concentrations occur in around half of children (30) thus a degree of overlap is not unexpected. In addition, children with IGF-I concentrations in the upper half of the normal range may not have been selected for GH stimulation tests by their clinician. Other limitations of the previous study included the fact that GH assay and stimulation test were not standardized. GH assay has been standardized in our current study and while we did use two different pharmacological stimulation tests (glucagon and arginine) they are known to achieve similar peak GH levels (4). Our study is still limited by the small number of GHD subjects and further studies with larger cohorts are required to validate the Random Forest algorithm we have generated.

A wide range of genetic mutations are now known to cause GHD either alone or as part of a wider spectrum of hypopituitarism (31). An approach to identify DNA mutations known to be causative of GHD in these patients may allow such a diagnosis without a GH stimulation test or RNA based test such as the one described in this paper. As such it is likely that analysis of targeted gene panels will become part of the diagnostic pathway in the future but, given that a genetic etiology is not identified in the majority of GHD patients, DNA based analysis is unlikely to replace pharmacological stimulation tests in the near future. There are also a smaller number of patients with acquired childhood GHD where GHD is caused by tumors, radiotherapy, head injury etc. While a whole exome/genome approach may aid in diagnosing congenital GHD it will not be helpful in children with acquired GHD. These children will still require pharmacological stimulation tests or a replacement such as our gene expression-based test for the diagnosis of GHD.

Gene ontology analysis of the genes identified as having predictive power from Boruta highlighted mTOR signaling which is a known component of the insulin and IGF-I signal transduction pathways (32, 33) and is involved in cell growth, differentiation and metabolism. Inositol phosphate metabolic processes were also identified with inositol known to be involved in glucose metabolism and carcinogenesis (34) and myoinositol having been previously linked to poor intra-uterine growth (35). The two genes identified within the confirmed Boruta gene set from this study and also from the top 10 genes of highest predictive power in our previous study were *NRXN1* and *PTGDS*. Neurexin 1 (encoded by *NRXN1*) is a neuronal presynaptic cell adhesion molecule involved in synaptogenesis and vesicular neurotransmitter release (36). Deletions and loss of function mutations in *NRXN1* are associated with neurodevelopmental and psychiatric phenotypes (36). *NRXN1* has been linked to circadian rhythm in a genome-wide association study (37) with pathogenic copy number variants in *NRXN1* also linked to increased body mass index (38). *PTGDS* encodes an enzyme which catalyses the conversion of prostaglandin H2 to prostaglandin D2. The $Ptgds^{-/-}$ mouse displays unilateral cryptorchidism (39) while low expression is linked to poor prognosis in endometrial cancer (40) and elevated levels linked to poor hair growth and androgenic alopecia in men (41).

In conclusion we have demonstrated a high degree of accuracy for diagnosis of childhood GHD utilizing a GE based test derived from a single blood sample expanding from our previous study to include short stature control subjects and the use of an RNA sequencing based approach. Further studies with greater numbers of patients are required to validate the random forest algorithm developed.

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 below: https://www.ncbi.nlm.nih.gov/geo/, GSE190502.

Ethics statement

The studies involving human participants were reviewed and approved by the Bradford Leeds Research Ethics Committee (Reference 18/YH/0226 IRAS ID 231325). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

The study was conceptualized and designed by PM, AS, and PC. RNA sequence analysis was undertaken by TG and IW. PM drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

PM has received honoraria from NovoNordisk. AS, PM, and PC have received speaker honoraria from Merck KGaA and a research grant from NovoNordisk.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1026187/full#supplementary-material

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COVID-19 pandemic phases and female precocious puberty: The experience of the past 4 years (2019 through 2022) in an Italian tertiary center

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Objective: Since the outbreak of COVID-19 pandemic, several centers of pediatric endocrinology worldwide have observed a significant increase in the number of girls presenting with precocious or early puberty. We aimed to compare the incidence rates of female precocious puberty before and during the different phases of COVID-19 pandemic.

Methods: We have retrospectively analyzed all the consultations recorded in the outpatient clinic database of the Endocrinology Unit of Bambino Gesù Children's Hospital, Rome, Italy, from the lockdown start in March 2020 up to September 2020, in comparison with the consultations recorded in the same months of 2019, 2021 and 2022. Age, height, weight, body mass index, Tanner's pubertal stage and bone age at presentation, birth weight, ethnicity, family history of central precocious puberty (CPP), maternal age at menarche, history of adoption were retrieved from clinical records. Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) both at baseline and after gonadotropin-releasing hormone (GnRH) stimulation, and basal estradiol levels were collected.

Results: In 2019, 78 girls with suspected precocious puberty were referred for endocrinological consultation, compared to 202 girls in 2020, 158 girls in 2021 and 112 girls in 2022. A significant increase in the proportion of girls diagnosed with rapidly progressive CPP was observed in 2020, compared to 2019 (86/202 *vs.* 18/78, $p<0.01$). In the following periods of 2021 and 2022, a gradual decrease in the number of cases of progressive CPP was evident, so much that the number of cases was not significantly different from that observed in 2019 (56/158 in 2021 and 35/112 in 2022, $p=0.054$ and $p=0.216$ respectively, compared to 2019).

Conclusions: Our research suggests that drastic lifestyle changes, such as those imposed by COVID-19 lockdown, and the consequent stress may affect the regulation of pubertal timing. The remarkable increase in CPP cases observed during the 2020 first pandemic wave seems to be reduced in 2021 and 2022, concurrently with the progressive resumption of daily activities. These data seem to support the hypothesis of a direct relationship between profound life-style changes related to the pandemic and the rise in precocious puberty cases.

KEYWORDS

precocious puberty, lockdown, COVID-19, pandemic, girls

1 Introduction

Puberty is the crucial transition process between childhood and adulthood, leading to full reproductive capacity (1). Female central precocious puberty (CPP) is defined as the onset of breast development before the age of eight years, due to the activation of the hypothalamic-pituitary-ovarian (HPO) axis (2). Puberty is a complex phenomenon, and factors modulating timing and/or tempo of puberty are not fully understood. It has been assumed that genetic, epigenetic and environmental factors, such as energy imbalance, exposure to endocrine disruptors or stressful events may trigger an earlier pubertal development (3, 4).

In the last century a trend toward earlier puberty was already observed (5–7). This phenomenon, known as "secular trend of puberty", has described a progressive reduction in the age at menarche, dropping from 17 years in the early-1800s to 13 years by the mid-1900s, with a further minor decline through the last three decades (5).

Recently, several centers of pediatric endocrinology worldwide, including ours, have observed a further significant increase in the number of girls presenting with precocious or early puberty since mid-2020 (8–21). During this period, corresponding to the first wave of COVID-19 pandemic, the Italian government imposed a strict lockdown across the country, in order to reduce the transmission rate and to avoid hospital bed saturation. Consequently, profound changes in everyday life occurred, such as school closures and the restriction of outdoor and team sports activities. Families were forced to stay at home, except for emergency reasons, with more opportunities for hypercaloric food consumption and overnutrition and the worsening of sedentary lifestyle. There was a significant rise of e-learning, extremely uncommon in primary schools before the pandemic. All these changes led to a larger daily use of electronic devices among children.

Given the growing worldwide evidence of an increase in female precocious puberty since the outbreak of COVID-19 pandemic, we aimed to investigate the evolution of this phenomenon before and during the different phases of the pandemic, from 2019 to 2022.

2 Materials and methods

2.1 Subjects

We retrospectively analyzed all the consultations for suspected precocious or early puberty recorded in the outpatient clinic database of the Endocrinology Unit of Bambino Gesù Children's Hospital, Rome, Italy from lockdown start in March 2020 to September 2020, in comparison with the consultations recorded in the same period of 2019, 2021 and 2022.

Consultations for premature thelarche in girls younger than 3 years were excluded. All subjects with suspected precocious puberty were observed for up to three months in order to reach the final diagnosis.

For each year, the subjects were further divided into subgroups based on the final diagnosis: transient thelarche (TT), non-progressive precocious puberty (NPP), CPP, or early puberty (EP). Subjects presenting with thelarche that disappeared during the 3-month observation period were assigned to the TT group. EP was defined as pubertal signs first appearing between 8 and 9 years, these girls were not further investigated.

The Institutional Review Board of 'Bambino Gesù Children's Hospital approved the study protocol.

2.2 Anthropometric data and medical history

Age, height (H), weight (W), body mass index (BMI), pubertal stage and bone age (BA) at presentation, birth weight, ethnicity, CPP family history, maternal age at menarche, history of adoption were retrieved from clinical records. H (cm) and W (kg) were also expressed as age and sex specific standard deviation score (SDS) according to the standard growth charts for the Italian population (22). Body mass index (BMI) was calculated as the ratio between W and H^2 and expressed as SDS. Birth weight was expressed also as SDS according to the Italian Neonatal Anthropometric Charts (23). Tanner's method was used to assess pubertal stages (24).

Questionnaires concerning physical activity, screen time and eating habits at the onset of pubertal signs were administered to all groups.

2.3 Laboratory measurements

Serum levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH) both at baseline and after gonadotropin-releasing hormone (GnRH) stimulation, and basal estradiol levels were collected, when available, among all subgroups except EP. GnRH stimulation test was performed by the i.v. administration of GnRH (Lutrelef; Ferring) at a dosage of 100 μg, with FSH and LH measurement at baseline and 30, 60 and 90 minutes after the injection. A basal LH level above 0.2 IU/l and/or a LH peak after GnRH infusion above 5 IU/l were considered diagnostic for CPP (2, 25). In the absence of one or both these criteria, subjects with slow pubertal progression were assigned to the NPP group.

2.4 Imaging

All subjects underwent pelvic ultrasound to assess uterine and ovarian characteristics. A uterine longitudinal diameter above 36 mm and the presence of the endometrium echo-pattern were considered signs of estrogenic stimulation, suggestive of precocious puberty.

An X-ray of the left hand and wrist was performed in all subjects to assess BA, according to the Greulich & Pyle method (26). Bone age advancement (years) was assessed as the difference between BA and chronological age.

Most subjects diagnosed with CPP (152 girls, 78%) underwent a magnetic resonance of the hypothalamus-pituitary area to rule out intracranial pathologies.

2.5 Statistical analysis

Data were expressed as mean ± SD when normally distributed and as median (interquartile range or IQR) for parameters with non-normal distribution, unless otherwise specified. Categorical variables were reported as number and percentage. The observed subjects were divided into four groups according to the year of evaluation (2019, 2020, 2021 or 2022). Each group was further divided in four subgroups according to the final diagnosis (TT, NPP, CPP or EP). Categorical variables were compared using chi-square (χ^2) test. ANOVA was applied to compare variables with normal distribution between more than two groups, while Kruskal–Wallis test was applied for variable with non-normal distribution.

Statistical analysis was performed with the statistical package SPSS v23 for Windows (SPSS Inc, Chicago, IL, USA) and a probability value of $p < 0.05$ was considered statistically significant.

3 Results

The sharpest increase of consultations was observed in 2020, with 208 subjects referred for suspected precocious or early puberty among a total number of 747 consultations in the period March-September 2020 (27.8%), in comparison with 85 subjects/1260 consultations in the same period of 2019 (6.7%). In 2021 there was still an increase in consultations for suspected precocious puberty, even if less pronounced than in 2020, with 166 subjects/1190 consultations (13.3%). A further reduction of consultations was observed in 2022, with 120 subjects/1380 consultations (8.7%).

Given the similarity in the number of boys observed throughout the years (7 subjects in 2019, 6 subjects in 2020, 8 subjects in 2021 and 8 subjects in 2022), we decided to further analyze only the female population of each considered period. Thirty-one girls were excluded because they were lost at follow-up after the first observation.

The study population consisted of 550 girls, divided as follows: 78 girls evaluated in 2019, 202 girls in 2020, 158 girls in 2021 and 112 girls in 2022. Figure 1 summarizes the design of the study and the results of data collection.

The number of consultations for suspected precocious or early puberty in girls was confirmed significantly higher in 2020 than in 2019 (202/747 equivalent to 27% in 2020 *vs.* 78/1260 equivalent to 6.2% in 2019, $p<0.01$). In 2020, the most evident increase in consultations was observed during the months following the lockdown (139/202 between June and September, equivalent to 72.8% *vs.* 63/202 between March and May, equivalent to 27.2%). In 2021 an initial downward trend was observed (158/1190, equivalent to 13.3%, $p<0.01$ *vs.* 2020), that became even further evident in 2022 (112/1380 equivalent to 8.1%, $p<0.01$ *vs.* 2020). This progressive downward trend led to a number of consultations in 2022 that was not significantly different from the number observed in 2019 (8.1% *vs.* 6.2% respectively, $p=0.06$) (Table 1).

CPP family history was positive in 28.7% of girls in 2020, in 24.1% in 2021 and in 31.3% in 2022, without significant differences with the 2019 population (35.9%). In total, ten girls had been adopted, 3 of them belonged to the NPP group and 7 to the CPP group.

The proportion of girls with rapidly progressive CPP was significantly higher in 2020, compared to 2019 (86/202 *vs.* 18/78, equivalent to 42.6% *vs.* 23.1%, $p<0.01$). In 2021, the number of cases of progressive CPP slightly decreased, compared to 2020 (56/158 *vs.* 86/202, equivalent to 35.4% *vs.* 42.6%, $p = 0.17$). In 2022, a further significant reduction in the number of cases of progressive CPP was observed compared to 2020 (35/112 *vs.* 86/202, equivalent to 31.3% *vs.* 42.6%, $p=0.04$). The number of cases observed in 2022 was not statistically different from the number of cases observed in 2019 (35/112 *vs.* 18/78, equivalent to 31.3% *vs.* 23.1%, $p=0.22$) (Figure 2).

Table 2 shows patients' characteristics according to the year of observation and final diagnosis.

No significant differences in anthropometric characteristics and laboratory parameters were found comparing the CPP subgroups of the four different years. The exceptions to this finding were a lower basal LH level in 2020 compared to 2022 (0.7 ± 0.98 IU/L in 2020 *vs.* 1.88 ± 1.99 IU/L in 2022, $p<0.01$) and a less evident BA advancement in 2020 compared to 2021 (1.32 ± 0.92 years in 2020 *vs.* 1.85 ± 1.17 years in 2021, $p=0.02$) (Table 3).

The majority of CPP girls (78%) underwent brain MRI study, none of them showed organic lesions related to CPP.

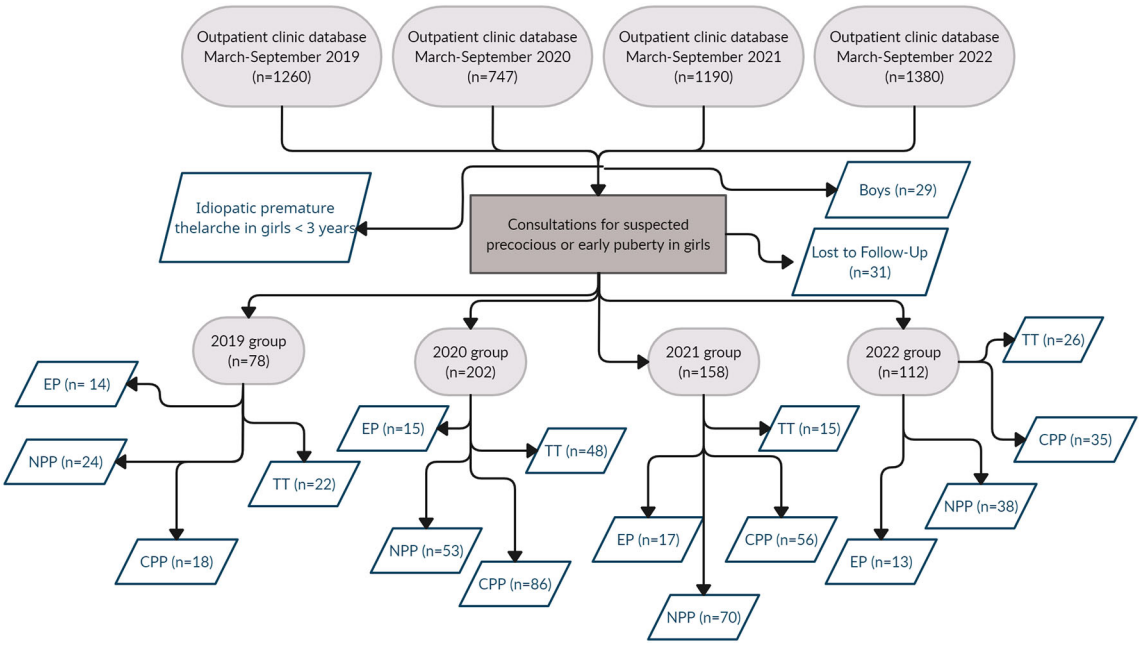

FIGURE 1
Flowchart summarizing the study design.

As regards to lifestyle, a significantly lower weekly physical activity was reported in the 2020 group compared to the 2019 and 2022 groups (median 1-2 h/week, IQR (0) in 2020 *vs.* 3-4 h/week, IQR (1-2 h/week to 5-6 h/week) in both 2019 and 2022, p<0.01) (Figure 3). In addition, the overall weekly time spent on electronic devices (as tablet, PC or smartphone) was considerably greater in the 2020 group than in 2019 and 2022 groups (median >20 h/week, IQR (0) in 2020 *vs.* 10-15 h/week, IQR (0) in 2019 and 5-10 h/week, IQR (1-5 h/week to 10-15 h/week) in 2022; p<0.01) (Figure 4). No significant difference in eating habits were evident among the groups.

4 Discussion

Our current data confirms the repeatedly reported, sharp increase in endocrinological consultations for suspected precocious or early puberty in girls, during the first waves of COVID-19 pandemic (8–21). As previously described, the increase in consultations was also reflected in an increase in CPP cases in 2020 compared to pre-pandemic values. Supporting the assumption of a different etiology between early and true precocious puberty, no difference in the number of cases of EP was observed throughout the four years.

The number of consultations for suspected precocious or early puberty in 2020 could have been affected by a selection bias due to the home confinement with elevated health anxiety that characterized the first phase of the pandemic. On the other hand, the significant increase of CPP cases in 2020 is supported by an objective diagnosis formulated by the same medical personnel among the different years.

For the first time, a gradual tendency towards a decrease of consultations and CPP cases during the evolution of the pandemic has been revealed, suggesting a downward trend of this phenomenon in concert with waning of the pandemic such that cases observed in 2022 were similar to the number of cases seen in 2019.

During 2021, the restrictive measures previously put in place to contain the pandemic were progressively relaxed and daily life

TABLE 1 Consultations for suspected precocious or early puberty recorded between March and September from 2019 to 2022, in comparison with the overall consultations recorded in the same period of the years.

	Visit for suspected precocious puberty between March-September			Total visit March-September
	March-May (%)	June-September (%)	Total (%)	
2019	37 (47.4)	41 (52.6)	78 (6.2)*	1260
2020	63 (27.2)	139 (72.8)	202 (27)*	747
2021	77 (48.7)	81 (51.3)	158 (13.3)*	1190
2022	48 (42.9)	64 (57.1)	112 (8.1)*	1380

*p<0.01 for 2020 vs. 2019, 2021 and 2022.

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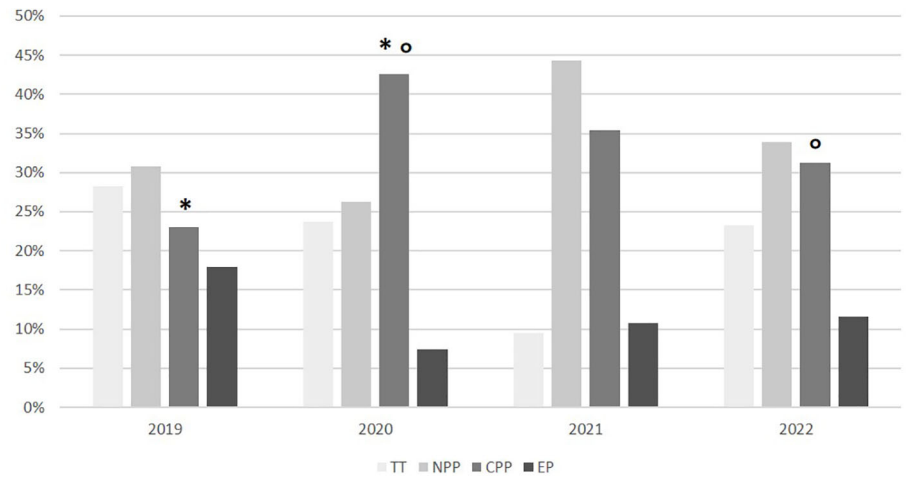

FIGURE 2
Subgroup distribution according on the final diagnosis in the different years. TT, transient thelarche; NPP, non-progressive precocious puberty; CPP, central precocious puberty; EP, early puberty. *p<0.01; °p<0.05.

activities returned to normal. Distant learning was gradually abandoned, at least in the primary schools, and children resumed face-to-face school activities. Group activities in leisure time and outdoor physical exercise resumed.

In a previous study (19), we correlated the increase of precocious puberty cases with home confinement, lack of physical exercise and the significant increase of daily screen time (both for studying and for leisure activities). These profound changes could have acted as stressors triggering the onset of puberty. The results of the present study seem to confirm the impact of lifestyle changes on pubertal timing.

Although there is no conclusive data on the association between poor physical activity and precocious puberty, a recent meta-analysis has confirmed that regular exercise training substantially increases adiponectin levels in obese children (27). Adiponectin, one of the most relevant adipokines secreted by mature adipocytes, has been demonstrated to suppress kisspeptin gene transcription

TABLE 2 Anthropometric parameters of study population according to year of observation and final diagnosis.

		Number (%)	Age (years)	Height SDS	Weight SDS	BMI SDS	BW SDS	BMI SDS – BW SDS
2019	TT	22 (28.2)	6.72 ± 0.81	0.57 ± 1.10	0.62 ± 1.07	0.55 ± 1.03*	-0.05 ± 1.12	0.57 ± 1.49
	NPP	24 (30.8)	7.34 ± 0.67°	0.99 ± 1.10	0.71 ± 1.15	0.42 ± 1.20	0.07 ± 1.05	0.30 ± 1.39
	CPP	18* (23.1)	7.02 ± 0.94	1.15 ± 1.01	0.87 ± 0.82	0.64 ± 0.61	-0.06 ± 0.85	0.65 ± 0.80
	EP	14 (17.9)	8.28 ± 0.39	0.84 ± 0.94	0.45 ± 1.01	0.20 ± 1.09	-0.09 ± 0.82	0.27 ± 1.41
2020	TT	48 (23.8)	6.89 ± 0.98	0.45 ± 0.98	-0.03 ± 0.86	-0.29 ± 0.90*	0.04 ± 1.29	-0.25 ± 1.38
	NPP	53 (26.2)	6.83 ± 0.84°	0.58 ± 1.01	0.50 ± 0.96	0.38 ± 0.96	-0.17 ± 1.08	0.58 ± 1.20
	CPP	86 (42.6)*°	7.05 ± 0.70	0.88 ± 0.94	0.40 ± 1.08	0.01 ± 1.80	-0.15 ± 1.01	0.24 ± 2.02
	EP	15 (7.4)	8.15 ± 0.35	0.52 ± 1.13	0.18 ± 1.10	0.02 ± 1.10	0.09 ± 1.09	-0.20 ± 0.99
2021	TT	15 (9.5)	6.55 ± 0.97	-0.01 ± 1.21	-0.12 ± 1.11	-0.08 ± 1.02	-0.01 ± 1.23	-0.27 ± 1.38
	NPP	70 (44.3)	7.13 ± 0.83	0.62 ± 0.93	0.69 ± 1.08	0.58 ± 1.15	-0.17 ± 0.99	0.77 ± 1.60
	CPP	56 (35.4)	7.27 ± 0.53	0.85 ± 0.99	0.57 ± 0.76	0.37 ± 0.77	-0.25 ± 1.23	0.64 ± 1.34
	EP	17 (10.8)	8.14 ± 0.44	1.00 ± 0.72	0.35 ± 0.69	-0.05 ± 0.84	-0.37 ± 1.12	0.12 ± 1.00
2022	TT	26 (23.2)	6.43 ± 0.94	0.41 ± 0.88	0.13 ± 0.94	-0.02 ± 0.89	-0.12 ± 0.94	0.08 ± 1.17
	NPP	38 (33.9)	7.26 ± 0.60°	0.70 ± 0.84	0.43 ± 0.91	0.22 ± 1.04	-0.05 ± 1.13	0.20 ± 1.22
	CPP	35 (31.3)°	7.30 ± 0.47	1.05 ± 1.17	0.60 ± 1.04	0.28 ± 1.11	-0.09 ± 1.40	0.28 ± 1.38
	EP	13 (11.6)	8.04 ± 0.67	0.66 ± 0.95	0.60 ± 0.88	0.54 ± 0.82	-0.22 ± 1.29	0.61 ± 1.04

CPP, central precocious puberty; EP, early puberty; NPP, non-progressive precocious puberty; TT, transient thelarche; BW, Birth Weight; BMI, Body Mass Index. *p<0.01; °p<0.05. Parameters are expressed as mean ± SD if not differently indicated.

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TABLE 3 Anthropometric characteristics and laboratory/instrumental parameter comparing the central precocious puberty (CPP) subgroups in the four different years.

CPP	2019	2020	2021	2022
Number (%)	18 (23.1)*	86 (42.6)*°	56 (35.4)	35 (31.3)°
Age (years)	7.02 ± 0.94	7.05 ± 0.70	7.27 ± 0.53	7.30 ± 0.47
Birth Weight SDS	-0.06 ± 0.85	-0.15 ± 1.01	-0.25 ± 1.23	-0.09 ± 1.40
Height SDS	1.15 ± 1.01	0.88 ± 0.94	0.85 ± 0.99	1.05 ± 1.17
Weight SDS	0.87 ± 0.82	0.40 ± 1.08	0.57 ± 0.76	0.60 ± 1.04
BMI SDS	0.64 ± 0.61	0.01 ± 1.80	0.37 ± 0.77	0.28 ± 1.11
BMI SDS – BW SDS	0.65 ± 0.80	0.24 ± 2.02	0.64 ± 1.34	0.28 ± 1.38
Basal LH (IU/L)	1.00 ± 1.51	0.52 ± 0.98*	1.13 ± 1.22	1.88 ± 1.99*
LH peak (IU/L)	19.49 ± 16.79	17.01 ± 14.02	22.48 ± 17.35	21.28 ± 14.27
17-beta-estradiol (pg/mL)	8.50 ± 10.02	16.54 ± 19.25	16.18 ± 18.26	24.23 ± 20.45
BA - CA	1.69 ± 0.75	1.32 ± 0.92°	1.85 ± 1.17°	1.82 ± 1.06
Uterine longitudinal diameter (mm)	42.04 ± 6.85	38.83 ± 8.02	41.30 ± 9.23	41.88 ± 7.65

Parameters are expressed as mean ± SD if not differently indicated. BMI, body mass index; BW, birth weight; BA, bone age; CA, chronological age. *p<0.01; °p<0.05.

and GnRH secretion by hypothalamic neurons, playing an inhibitory role in the onset of puberty (28, 29).

Beyond the physical benefits of exercise, several studies reported a positive association between physical activity and psychological well-being in children and adolescents. A sedentary lifestyle has been related to both depression and lower life satisfaction and happiness, while promoting physical activity and decreasing sedentary behavior might protect mental health (30). Early studies (31, 32) suggested that psychological stress itself (due to insecure bonds with parents or parental conflicts) might modify pubertal timing. A recent study reported that anxiety and other internalizing symptoms in pre-pubertal girls are associated with early pubertal onset, independently from maternal education anxiety, BMI, and ethnicity (33).

Several studies have recently investigated the effects of exposure to electromagnetic fields on melatonin (34–37). Exposure to electromagnetic fields has been associated with decreased melatonin production *in vitro*, as well as with a decreased pineal and plasma melatonin and its urinary metabolites (35). Nighttime serum melatonin levels are highest in infants and young children and decrease progressively by 80% throughout childhood and adolescence, nocturnal melatonin levels drop in parallel with sexual maturation (38, 39). Animal models have also shown that a reduction in melatonin may accelerate pubertal development (40) and that the administration of melatonin suppress GnRH secretion (41). A recent study performed on immature female rats differentially exposed to a light spectrum predominantly emitted by LED (light-emitting diode) screens, showed a faster pubertal maturation in rats bathed with the

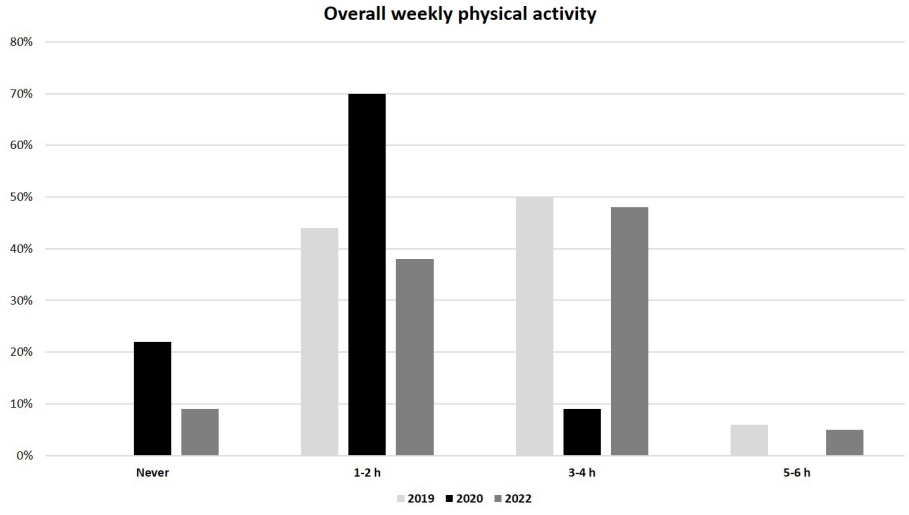

FIGURE 3
Overall weekly physical activity in 2019, 2020 and 2022 populations.

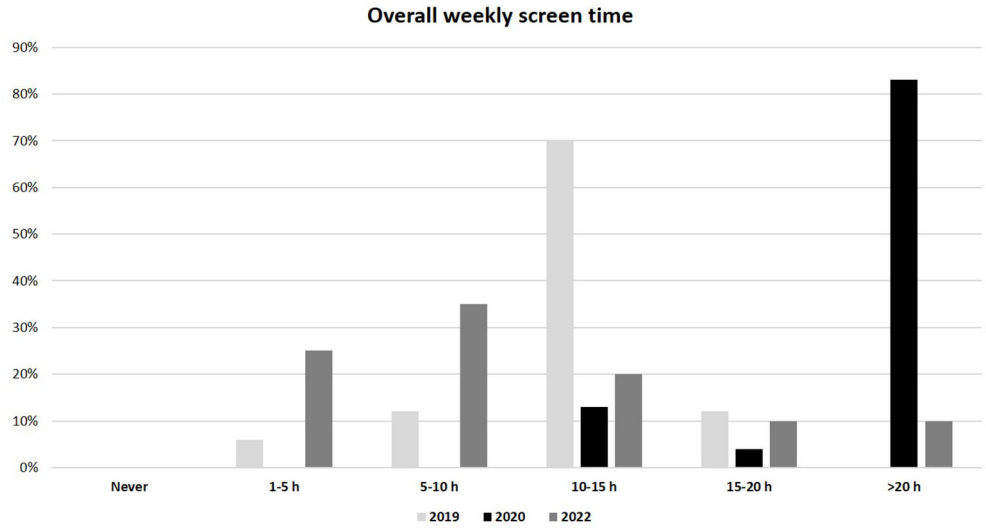

FIGURE 4
Overall weekly use of electronic devices (as tablet, PC and smartphone) in 2019, 2020 and 2022 populations.

blue-tinged light for longer bouts (42). The combination of this data suggests that a greater use of electronic devices leads to a reduction in melatonin levels, which in turn triggers the endocrine changes culminating in the earlier onset of puberty (43).

Another study reported more frequent late bedtime, sleep disturbances, excessive somnolence, sleep breathing disorders and sleep-wake transition disorders in girls diagnosed with CPP during the Italian lockdown (15).

Published data analyzing the impact of overweight and obesity on the rise of CPP cases are conflicting (8, 13, 44, 45). Interestingly, we did not find any significant difference in BMI SDS at CPP diagnosis across the four years of observation, suggesting that overnutrition and overweight do not represent determining factors in this context.

All the mentioned factors (inactivity, increased screen time, sleep disturbances, and stress) may have contributed to the sharp increase in CPP cases, acting directly on the HPO axis. The retrospective design of the study does not allow identifying which factor predominates over the others. Indeed, the speed and reversibility of the phenomenon and the absence of differences in the anthropometric characteristics of the groups (in particular, BMI unchanged over the years) allows us to rule out already known risk factors for CPP (such as endocrine disruptors, obesity, or epigenetic factors). In support of this hypothesis, we observed lower basal LH levels and the less evident BA advancement in the 2020 CPP cases, compared to 2019. This could suggest that life-style changes can only act as weaker triggers of GnRH secretion with a transient effect on pubertal timing.

A single study from Korea described an almost doubled CPP incidence in 2021, in comparison with 2016, with a concurrent increase in the proportion of boys (19.55% *vs.* 9.21%) (11). As in the majority of the published studies, we reported an increase of CPP cases uniquely in girls. This fact seems to confirm that male CPP, in its rarity, is mostly related to organic disorders and/or genetic factors and less influenced by environmental changes.

We are aware that the major limitation of this study is its retrospective design, which did not allow us to obtain more data on factors potentially influencing GnRH secretion, but to our knowledge, this is the first study that describes a progressive downward trend in CPP cases during the post-pandemic period in 2022 to near pre-pandemic levels.

In conclusion, the sharp increase of CPP cases in girls during the first pandemic wave in mid-2020 seems to give way to a gradual downward trend, concurrently with the easing of the restrictive measures, returning to the pre-pandemic incidence of CPP in 2022. This suggests that the drastic lifestyle changes, as lack of physical exercise, increased screen time, sleep disturbances, and stress, may represent weak and reversible triggers on the central "biological clock" controlling timing and tempo of puberty.

Data availability statement

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

Ethics statement

The studies involving human participants were reviewed and approved by Bambino Gesù Children's Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

CB and MCa conceptualized and designed the study. GB, LC, LP, TT, and MCh collected data. LC and MCh performed statistical analysis. CB, LC, and MCh drafted the initial manuscript, and

reviewed the manuscript. MCa and CB revised the manuscript. 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.

Publisher's note

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A novel DEAH-box helicase 37 mutation associated with differences of sex development

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Objective: To determine the genetic etiology of a family pedigree with two patients affected by differences of sex development (DSD).

Methods: Assess the clinical characteristics of the patients and achieve exome sequencing results and *in vitro* functional studies.

Results: The 15-year-old proband, raised as female, presented with delayed puberty and short stature associated with atypical genitalia. Hormonal profile showed hypergonadotrophic hypogonadism. Imaging studies revealed the absence of a uterus and ovaries. The karyotype confirmed a 46, XY pattern. Her younger brother presented with a micropenis and hypoplastic scrotum with non-palpable testis and hypospadias. Laparoscopic exploration was performed on the younger brother. Streak gonads were found and removed due to the risk of neoplastic transformation. Post-operative histopathology showed the co-existence of Wolffian and Müllerian derivatives. Whole-exome sequencing identified a novel mutation (c.1223C>T, p. Ser408Leu) in the Asp-Glu-Ala-His-box helicase 37 gene, which was found to be deleterious by *in silico* analysis. Segregation analysis of the variant displayed a sex-limited, autosomal dominant, maternal inheritance pattern. *In vitro* experiments revealed that the substitution of 408Ser by Leu caused decreased DHX37 expression both at the mRNA and protein levels. Moreover, the β-catenin protein was upregulated, and the p53 protein was unaltered by mutant *DHX37*.

Conclusions: We described a novel mutation (c.1223C>T, p. Ser408Leu) of the *DHX37* gene associated with a Chinese pedigree consisting of two 46, XY DSD patients. We speculated that the underlying molecular mechanism might involve upregulation of the β-catenin protein.

KEYWORDS

46, XY differences of sex development, family pedigree, DEAH-box helicase 37, whole-exome sequencing, β-catenin

1 Introduction

According to the 2006 Chicago consensus statement, differences of sex development (DSD) can be classified into the following: sex chromosome DSD; 46, XY DSD; and 46, XX DSD (1). The occurrence of 46, XY DSD is primarily a consequence of genetic variants leading to disorders of testicular development or defects in androgen biosynthesis or action (1). According to the degree of testicular differentiation, interruption of the male sex-determination pathway causes a phenotype of gonadal dysgenesis (GD) in 46, XY individuals, including partial and complete forms. Embryonic testicular regression syndrome (ETRS), characterized by atypical genitalia and lack of gonadal tissue on one or both sides, has been considered part of the clinical spectrum of 46, XY GD (2). The incidence of 46, XY DSD is estimated to be 1:20000 births and of 46, XY GD around 1:100000 births (3). However, the incidence of DSD or GD may be underestimated due to the rarity of some of the conditions and lack of definitive clinical diagnosis. Besides complete clinical data, detailed genetic analyses, which have been challenging, are pivotal in the diagnosis of DSD. To date, there are at least 18 genes that have been found to be related to 46, XY GD (4). The pathogenic variants in *SRY* (MIM 480000), *NR5A1* (MIM184757), and *MAP3K1* (MIM 600982) are the three most prevalent causes, in total accounting for 40% of individuals with 46, XY GD (4). Pathogenic variants in other sex-determining genes, such as *SOX9* (MIM 608160), *SOX8* (MIM 605923), *GATA4 (*MIM 600576), *DMRT1* (MIM 602424), *FOG2* (MIM603693), *WT1* (MIM 607102), *DHH* (MIM 605423), *CBX2* (MIM 602770), and *DMRT3* (MIM 614754), are found in a small portion of cases (3, 5–7). Therefore, the etiology of the majority of individuals with DSD remains unclear.

Recent studies have identified pathogenic variants in the DEAH-box RNA helicase *DHX37* as a new cause of 46, XY GD and ETRS (8–11). DHX37 is an ATP-dependent RNA helicase and is required for ribosome biogenesis (12). It has been assumed that mutation in the *DHX37* gene might impair ribosome biogenesis; therefore, DSD associated with defective *DHX37* was supposed to be a new ribosomopathy (13). Other biological functions of DHX37 independent of ribosome biogenesis have also been reported. For example, studies of zebrafish carrying mutant *DHX37* demonstrated that *DHX37* physically interacted with the *GlyR α1*, *α3*, *and α4a* subunits, and in mutants the expression of the above transcripts were decreased. Notably, there was no difference in the amount of 18S and 28S rRNA between the wild-type and mutant zebrafish, indicating little effect on ribosome biogenesis (14). Other evidence included genome-wide CRISPR screens identifying DHX37 as an important regulator of human CD8 T-cell activity (15). McElreavey et al. briefly summarized the published *DHX37* pathogenic variants and tried to demonstrate how these variants caused DSD (13). The most common pathogenic variant is the p.Arg308Gln amino acid change (8–11); however, there is no evidence for certain signaling pathways underlying the pathogenesis of DSD caused by *DHX37* variants. In our study, we identified a novel mutant of *DHX37* (c.1223C>T, p.Ser408Leu), which was associated with 46,XY DSD in a Chinese pedigree. *In silico* modeling predicted that the mutation of c.1223C>T would be deleterious to the DHX37 protein. Notably, we proved that the perturbation of *DHX37* led to the upregulation of the β-catenin protein, which might underly the mechanism of DSD caused by defective *DHX37*. Our findings extend the variants associated with DSD and highlight the phenotype spectrum associated with *DHX37*. We also provided evidence that DSD caused by defective *DHX37* may have a link with the activation of the Wnt/β catenin pathway.

2 Materials and methods

2.1 Subjects

Clinical data of two non-twin siblings affected by 46,XY DSD in a Chinese pedigree were collected. Data collected included gender raised as, age at presentation, gynecological examination, hormone profile (follicle-stimulating hormone, luteinizing hormone, testosterone, and anti-Müllerian hormone), karyotyping, family history of DSD, and consanguinity. Abdominal/inguinal ultrasound or urinary CT was performed where specified. A removed gonad stained with hematoxylin and eosin (HE) for histological analyses was provided by the Department of Pathology in our hospital. Written informed consent was obtained from all family members. The Ethics Committee approved this study, including the chromosomal and molecular biology analyses (Institution Review Board of Guizhou Provincial People's Hospital [2021(No. 3)].

2.2 Whole-exome sequencing, data analyses, and *in silico* prediction

Genomic DNA was extracted from peripheral blood using standard procedures (MagPure Buffy Coat DNA Midi KF Kit, MAGEN). Whole-exome sequencing (WES) of the genomic DNA was performed, and a blood sample of the proband was sequenced with PE100+10 on MGISEQ-2000. The sequenced data was aligned to the human genome reference (hg19) using the BWA (Burrows Wheeler Aligner) Multi-Vision software. After alignment, the output files were performed sequencing coverage and in-depth analyses of the target region, single-nucleotide variants (SNVs), and INDEL calling. GATK software was used to detect SNVs and indels, which were filtered and estimated *via* multiple databases, including HapMap, NCBI dbSNP, 1000 human genome dataset, and a database of 100 Chinese healthy adults. We used the Human Gene Mutation Database (HGMD) to screen mutations reported in the published studies. The pathogenic effect of the variant was predicted by three software programs (Polyphen2, Mutation Taster, and PROVEAN) and assessed under the protocol issued by ACMG (16). The potential pathological variant identified by WES was then validated by Sanger sequencing. To predict the molecular consequences of the variant, the homology models of the wild-type (WT) and mutant DHX37 were generated using SWISS-MODEL with the most suitable model (Seq Identity=100% and coverage: 3-1157). To predict the stability of the protein, the protein stability prediction tool I-Mutant (http://folding.biofold.org/i-mutant/) was used.

2.3 *In vitro* functional studies of DHX37 mutant

2.3.1 Construction of plasmids

Complementary DNA (cDNA) encoding WT DHX37 was cloned into the digested pcDNA3.1 vector, producing pcDNA3.1-DHX37-WT. The single mutation (p.S408L) was inserted using a Site-Directed Mutagenesis Kit (Vazyme, China), generating pcDNA3.1-DHX37-S408L. The entire coding sequence of both4plasmids was certified by direct sequencing prior to functional studies.

2.3.2 Cell culture, transfection, and functional analyses

A human Sertoli cell line (iCell-0085a, iCell Bioscience Inc., China) was used for molecular studies. The cells were cultured in a special culture medium for human Sertoli cells (iCell-0085a-001b, iCell Bioscience Inc., China) and passaged with standard procedures. The empty expressing vector, mutant, and WT constructs were transiently transfected into the Sertoli cells using the Lipofectamine 3000 Transfection Reagent (Invitrogen, USA) according to the manufacturer's protocol. Cell viability was observed with Cell Counting Kit-8 (KGA317, KeyGen Biotech, China). The apoptotic rate of cells was evaluated by flow cytometry (FCM) with Annexin V-FITC/PI kit (AP101-100-kit, MultiSciences Biotech, China). Western blot analysis and quantitative real-time PCR were performed as previously described (17). The primary antibody p53 (1:500) was from Affinity Biosciences (AF0879), β catenin (1:500) was from Servicebio (GB11015), DHX37 (1:500) was from Bioss (bs-14320R), and β-actin (1/2000) and GAPDH, which was used as loading control, were from TransGen Biotech (β-actin:HC201; GAPDH : HC301). The horseradish peroxidase-conjugated secondary antibody (1:2,000) was from Beijing Zhongshan Jinqiao Biological Technology (anti-Rabbit ZB-2301; anti-Mouse: TA-09). Protein band densities were quantified using the Image J program. The primers for qPCR were as follows: β-actin: 5'-TGGCACCCAGCACAATGAA-3' and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3'; DHX37:5'- CGGCGCTACAACATCAAGG-3' and 5'-CTTCTTCCCCGGTAGAACGAG-3'.

2.3.3 Immunofluorescence cell staining

Cultured Sertoli cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with 0.1% Triton-X-100 for 20 min. Then, the cells were washed three times with PBS and blocked with 5% BSA for 30 min at 37°C. Cells were incubated with the anti-DHX37 antibody (1:200 dilutions, bs-14320R, Bioss, China) overnight at 4°C, followed by the secondary goat anti-rabbit IgG-Cy3 antibody (1:200 dilutions, AS007, ABclonal, China) incubated for 30 min at 37°C. Nuclear staining of the cells involved using DAPI (KGA215-50, KeyGen Biotech, China) for 3 min at room temperature. Images were captured by fluorescence microscopy (Olympus, Japan).

2.4 Statistical analysis

GraphPad 9.0 (Prism, USA) was used for data analysis. Quantitative data with normal distribution was presented as means ± SD from at least three independent experiments. Statistical analysis was performed with the use of a one-way ANOVA followed by multiple comparisons with a *post hoc* Tukey's test. A p value of less than 0.05 was considered to be statistically significant.

3 Results

3.1 Clinical characteristics of the pedigree

The 15-year-old proband (III:1), raised as female, presented with complaints of short stature, with no signs of puberty and menstrual bleeding. At the time of admission, her height was 139.5cm, and her weight was 34.8kg. She had normal intellectual function and facial appearance, with development of both breasts at Tanner I and absence of pubic and axillary hair. Gynecological examination revealed poorly developed labia, clitoral hypertrophy, and absence of vaginal opening. An abdominal and pelvic ultrasound did not show the ovaries and uterus. Inguinal ultrasound ruled out the presence of testes in the inguinal region. The hormonal profile revealed low levels of both total testosterone (0.4ng/mL, 4.94-32.01ng/ml) and anti-Müllerian hormone (AMH) (0.02ng/ml, 0.96–13.34 ng/mL), but elevated levels of follicle-stimulating hormone (FSH) (73.34 U/L, 0.95-11.95 U/L) and luteinizing hormone (LH) (19.32 U/L, 0.57-12.07 U/L). The karyotype was mapped, demonstrating a 46, XY pattern (Figure 1A).

Further investigation revealed that one of the younger brothers also had 46, XY gonadal dysgenesis. The boy (III:2) was nine years old when admitted to our department. Physical examination revealed a micropenis, hypospadias, and hypoplastic scrotum with non-palpable testis. The hormonal profile revealed a low testosterone level of 0.22 ng/mL (4.94-32.01ng/ml) and a slightly elevated level of FSH 17.27 U/L (0.95-11.95 U/L). The LH level was 1.15 U/L (0.57-12.07 U/L), and the AMH was not examined. The karyotype was 46, XY (Figure 1B). A CT scan (Figure 2A) of the urinary system showed an empty scrotum and a small penis. Bilateral streak gonads were found upon laparoscopy. In respect of the high risk of gonadal tumors in DSD patients (18), the streak structure was removed, and post-surgical histopathology demonstrated a mixture of epididymis- and fallopian tube-like structures on both sides, as well as remnants of the ductus deferens on the left side (Figure 2B). The clinical characteristics of the two affected siblings are listed in Table 1.

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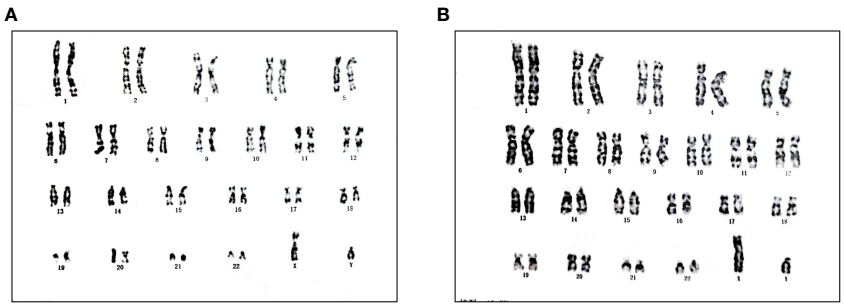

FIGURE 1
Karyotype of III:1 **(A)** and III:2 **(B)**.

We confirmed that the two younger sisters (III:3, III:4) had the 46, XX karyotype (Supplementary Figures 1A, B), and the youngest brother had 46, XY (III:5) (Supplementary Figure 1C); each of the three children had a normal facial appearance, intellectual development, and age-appropriate development of external genitalia.

3.2 Whole-exome sequencing identifies *DHX37* mutation

Family pedigree chart is shown in Figure 3A. A variant (c.1223C>T; p.Ser408Leu) in exon 9 of the *DHX37* gene was obtained in the sample of the proband by exome sequencing. The mutation was not present in 1000 Genomes, ESP6500, ExAC, GnomAD, and GnomAD-EAS. Three programs (Polyphen-2, Mutation Taster, and PROGEAN) predicted this mutation would be deleterious to the protein function (Table 2). The variant was validated by Sanger sequencing in all family members; the representative sequencing results are shown in Figure 3B. Both the proband (III:1) and the clinically affected brother (III:2) carried the heterozygous variation. Moreover, their asymptomatic mother (II:2) and one of the younger sisters (III:4) carried the same variation. The rest of the family members (Subjects I:1, I:2, II:1, II:3, II:4, II:5, III:3, and III:5) had no variation.

We further investigated how the p.Ser408Leu mutation affected the DHX37 protein structure. The DHX37 protein (NP_116045) comprises 1157 amino acids (AAs) and four main domains, including two RecA-like domains, which are the helicase core domains (RecA1: ATP-binding DEAH-box helicase, RecA2: C-terminal helicase), helicase-associated 2 domain (HA2), and oligonucleotide/oligosaccharide-binding fold domain (Figure 4A). The conserved motifs of the helicase core region are involved in RNA substrate interaction, ATP binding, and hydrolysis, as well as the coordination of the unwinding activity (9–12). The variant

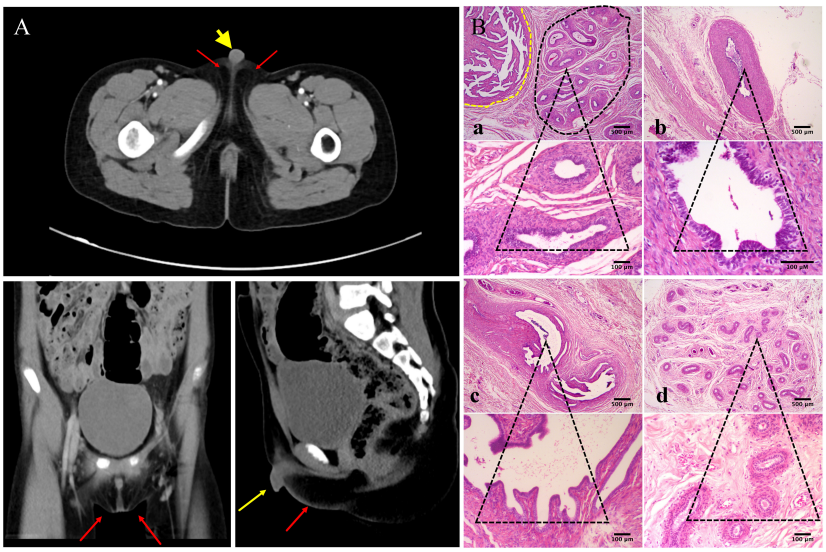

FIGURE 2
Urinary CT scan and post-surgical histopathology of the gonads of III:2. **(A)** CT scan of the urinary system shows empty scrotum (red arrows) and small penis (yellow arrow). **(B)** Histologic analysis of gonad samples from III:2. No gonadal tissue was observed; however, fallopian tube-like and epididymal-like structures were present in both gonads of the patient. **(a)** Fallopian tube-like (circled by yellow dashed line) and epididymal-like structures (circled by black dashed line) on the left side were seen in the same microscopic field; **(b)** remnants of the ductus deferens were also present on the sample from the left side; **(c)** fallopian tube-like and **(d)** epididymis-like structures on the right side. The lower panel shows indicated tissues on the upper panel at higher magnification. Size bars are indicated for each panel. Staining was performed with hematoxylin–eosin.

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TABLE 1 Clinical characteristics of III:1 and III:2.

	Gender raised as	Karyotype	Age at presentation	Diagnosis	External genitalia	Internal genitalia	Gonadal histology	LH(U/L) Reference range: 0.57-12.07	FSH(U/L) Reference range: 0.95-11.95	T(ng/ml) Reference range: 4.94-32.01	AMH(ng/ml) Reference range: 0.96-13.34
III:1	Female	XY	15	46,XY TRS	Poorly developed labia, clitoral hypertrophy, absence of vaginal opening	No mullerian structures, no gonads present	NA	19.32	73.34	0.4	0.02
III:2	Male	XY	10	46, XY PGD	Micropenis, hypoplastic scrotum with non-palpable testis, hypospadias	No mullerian structures, no gonads present	Fallopian tube- and epididymis-like structures on both sides, remnants of the ductus deferens on the left side	1.15	17.27	0.22	NA

NA, not available.

c.1223C>T (p.Ser408Leu) is located in the helicase ATP-binding domain and falls within the Motif III (Figure 4B), which has been implicated in the coordination of ATP hydrolysis and unwinding (9–12). Notably, the affected amino acid residue Ser408 is highly conserved across different species (Figure 4B), suggesting its structural and functional importance. Homology models of DHX37 generated by SWISS-MODEL showed that Ser408 and Thr410 and Val273 and Gly275 were linked by hydrogen bonds in the wild type (Figure 4C). After the mutation, the hydrogen bonds between Ser408 and Thr410 disappeared, and large side chains were introduced (Figure 4D). Moreover, as the serine acid is hydrophilic and polar, while the leucine is hydrophobic and non-polar, we also investigated the hydrophobicity of the protein region by use of Kyte and Doolittle hydropathy plots (Figures 4E, F); we found that the p.Ser408Leu variant caused the increased hydrophobicity in the region between codons 400 and 420. In this work, we found this mutation increased the stability of the protein by using the protein stability prediction tool, which demonstrated the pathogenic role of the p.Ser408Leu mutant.

3.3 *In vitro* functional studies

To further investigate the pathogenesis of the p.Ser408Leu mutation, the pcDNA3.1-WT and pcDNA3.1-S408L plasmids were constructed and separately transfected into human Sertoli cells. To first ascertain the localization of DHX37, we performed immunofluorescence in the Sertoli cells by using an antibody against DHX37. This revealed that DHX37 predominantly localized to nucleoli, although protein was also observed in the cytoplasm. Both wild-type and mutant DHX37 exhibited the same cellular localization (Figure 5A). DHX37 expression in cells transfected with either pcDNA3.1-WT or pcDNA3.1-S408L was analyzed by real-time PCR and Western blot. As shown in Figures 5B, C compared with the cells transfected with pcDNA3.1-WT, those with pcDNA3.1-S408L displayed decreased DHX37 expression both at the mRNA and protein levels.

Classically, impaired ribosome biogenesis triggers nuclear stress, which leads to cell apoptosis partly through stabilization of the tumor suppressor p53 (19). Moreover, nuclear stress was recently found to activate WNT/β-catenin signaling (20). Therefore, the effect of mutant *DHX37* in p53 and β-catenin signaling was examined. As shown in Figure 5C, there is no difference in p53 expression among cells transfected with empty expressing vector, wt-*DHX37*, or mutant *DHX37*. Interestingly, transfection with wt-*DHX37* led to a significant decrease of the β-catenin protein, which was rescued by the mutant *DHX37*. We further examined the effect of DHX37 on cell apoptosis and proliferation. As shown in Figure 5D, compared with the cells transfected with empty expressing vector, those with wt-*DHX37* or mutant *DHX37* exhibited an increased apoptosis rate. Furthermore, cells that expressed wt-*DHX37* showed an even higher apoptosis rate than the mutant. As demonstrated in Figure 5E, overexpression of both wt-*DHX37* and mutant-*DHX37* decreased cell proliferation, but there was no difference between the two groups.

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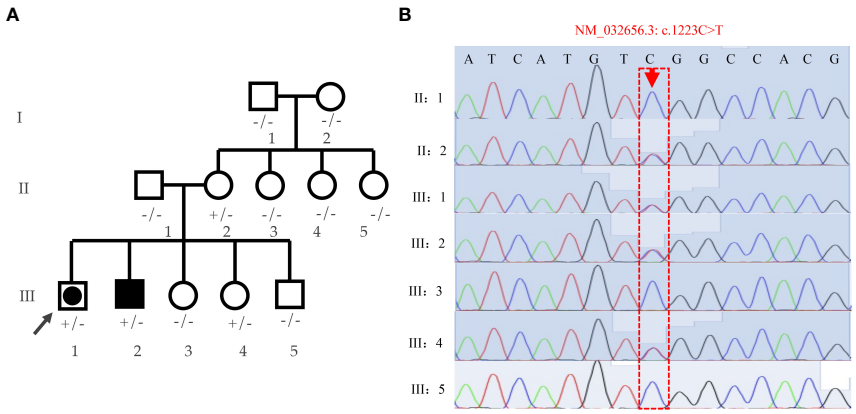

FIGURE 3
Analyses of the family pedigree with two cases affected by 46,XY DSD. **(A)** Family pedigree. Closed symbols represent affected individuals. The affected male (46, XY males) is indicated by a closed square and the affected individual raised as female (46,XY females) is shown by a large, closed circle within a square. Whole-exome was performed on the proband (indicated by black arrow), and Sanger sequencing were verified on all family members; genotypes are labeled on the chart. +/- heterozygous state; -/- homozygous state for wild-type allele. **(B)** Verification of the S408L mutation by means of Sanger sequencing in the pedigree.

TABLE 2 Prediction of the pathogenicity of S408L by a range of soft programs.

Program	Score	Prediction
PolyPhen-2	0.999	probably damaging
Mutation taster	0.999999999721836	disease causing
PROVEAN	-5.824	deleterious

4 Discussion

We identified a heterozygous c.1223C>T mutation (p.Ser408Leu) in exon 9 of *DHX37* in a pedigree affected by 46,XY GD by using WES. The mutation was not present in 1000 Genomes, ESP6500, ExAC, GnomAD, or GnomAD-EAS. Moreover, the p.Ser408Leu substitution is considered probably damaging (0.999) by PolyPhen2, disease-causing (0.999) by Mutation Taster, and has a PROVEAN score of -5.824 (deleterious). In the DEAH-box family of proteins, Ser408 is a highly conserved residue across various species. It falls within Motif III, which is known to couple ATPase and unwinding activity (9–12). The mutation p.Ser408Leu may affect the alignment of the two RecA-like domains responding to NTP binding or fail to assemble the NTP active site responding to nucleic acid binding; therefore, it may impair the coordination of ATP hydrolysis and unwinding (21). We also provided evidence that the p.Ser408Leu variant changed the polarity and stability of the DHX37 protein. Moreover, *in vitro* studies demonstrated that the DHX37 mRNA and protein decreased significantly in cells carrying mutant *DHX37*. Importantly, β-catenin was upregulated by mutant *DHX37*, which may contribute to the pathogenesis of 46, XY DSD caused by defective *DHX37*.

To date, *DHX37* variants associated with 46, XY DSD have ben transmitted either maternally or *de novo*, except for c. G 923A (p. Arg308Gln) (10) and c.C1430T (p. Thr477Met) (9), each of which in a family were reported to be inherited by the proband from their fertile father. In our current study, the *DHX37* variant was delivered from the asymptomatic mother. Moreover, one of the proband's sisters (III4), who carried the same variant, exhibited a normal phenotype, demonstrating a sex-limited inheritance mode. McElreavey et al. (13) showed that DHX37 expression was higher in male gonads than female, suggesting its important role in regulating the development of male gonads. In our study, with the use of the human Sertoli cell line, we identified the presence of DHX37, which was predominantly localized to the nucleus. The protein was also present in the cytoplasm, consistent with the process of ribosome formation taking place initially in the nucleolus and then in the cytoplasm (13). The expression of DHX37 in human Sertoli cells was also observed by McElreavey et al. (11, 13). However, in another study, while DHX37 was seen in Leydig cell cytoplasm and germ cells at different stages of maturation, rare Sertoli cells displayed a weak and focal cytoplasmatic stain (10). The expression pattern of DHX37 may vary depending on the developmental stages of the testes. In the fetal gonads of mice and humans, DHX37 is expressed only in the somatic cell lineages but not in germ cells (11). In adult human testes, however, the protein is observed in spermatogonia (11). Interestingly, it was found that as the cells differentiated from spermatocytes to spermatids, the protein exhibited a progressive

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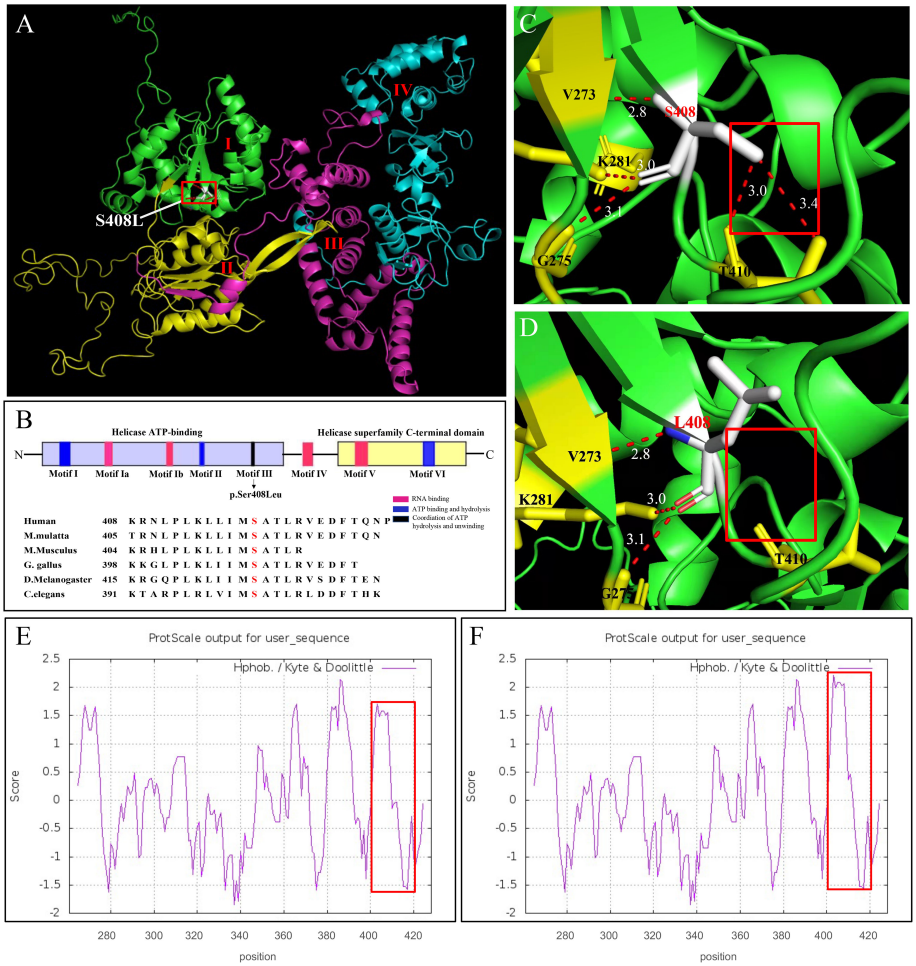

FIGURE 4
In silico modeling of DHX37. **(A)** Functional domains of a homology model of the DHX37 protein. The protein has four functional domains, which are color-coded and labeled. I: ATP-binding DEAH-box helicase (green), II: C-terminal helicase (yellow), III: helicase-associated 2 domain (purple), and oligonucleotide/oligosaccharide-binding-like domain (blue). The variant p.Ser408Leu is located in the helicase ATP-binding domain. **(B)** Schematic protein structure of the helicase core region, i.e., the helicase ATP-binding domain and the helicase superfamily C-terminal domain. Colors represent the main helicase functions. Sequence alignment shows the conservation of the amino acid residue S408 across different species. The variant p.S408L falls within the Motif III, which is implicated in the coordination of ATP hydrolysis and unwinding. A zoomed-in view of protein model with residue S408 **(C)** and L408 **(D)**. S408 and T410 and V273 and G275 were linked by hydrogen bonds in the wild type. After the mutation, the hydrogen bonds between S408 and T410 disappeared. N-, N terminus; -C, C terminus. Kyte and Doolittle hydropathy plot of the DHX37 protein **(E)** before and **(F)** after p.S408L mutation. A score >0 means hydrophobic and <0 means hydrophilic. Higher positive values indicate greater hydrophobicity. The mutation caused increased hydrophobicity in the region between codons 400 and 420, as outlined by red boxes.

condensation around the nucleus (10). The unique expression pattern of the DHX37 protein may imply its important role in testis development and maintenance of testicular function.

According to previous studies, there is a poor genotype–phenotype correlation in the 46, XY DSD patients associated with *DHX37* variants. Even for those who carry the same pathogenic mutation of DHX37, the genital phenotype can range from predominantly female to male. The degree of virilization depends on the duration of the functional testis before it subsequently vanishes. Although it happens occasionally, there are male carriers who have typical external genitalia and preserved fertility, suggesting sufficient functional testicular tissue for the development of the external genitalia and to support spermatogenesis (9, 10). Unknown genetic modifiers may prevent the appearance of phenotype in individuals with pathogenic *DHX37* variants. In this study, the proband was raised as female with atypical external genitalia and absence of gonads in either side, while her younger brother, who carried the same *DHX37* variant, was raised male with a micropenis and had partially developed internal ducts consisting of a mixture of Wolffian (epididymis-like structures, vas deferens) and Müllerian ducts (fallopian tube-like structures). The proband was clinically diagnosed as 46, XY ETRS, while her younger brother was diagnosed as 46, XY PGD, reinforcing the heterogenicity of 46, XY DSD and that 46, XY GD and ETS form part of the same phenotypic spectrum and share the same etiological mechanism.

Considering the role of DHX37 in ribosome biogenesis, DSD caused by *DHX37* defects is suggested to be a kind of ribosomopathy (13). Exactly how mutant DHX37 proteins cause a highly specific human developmental disorder is confusing, since it is widely expressed and involved in a basic cellular function. McElreavey, K

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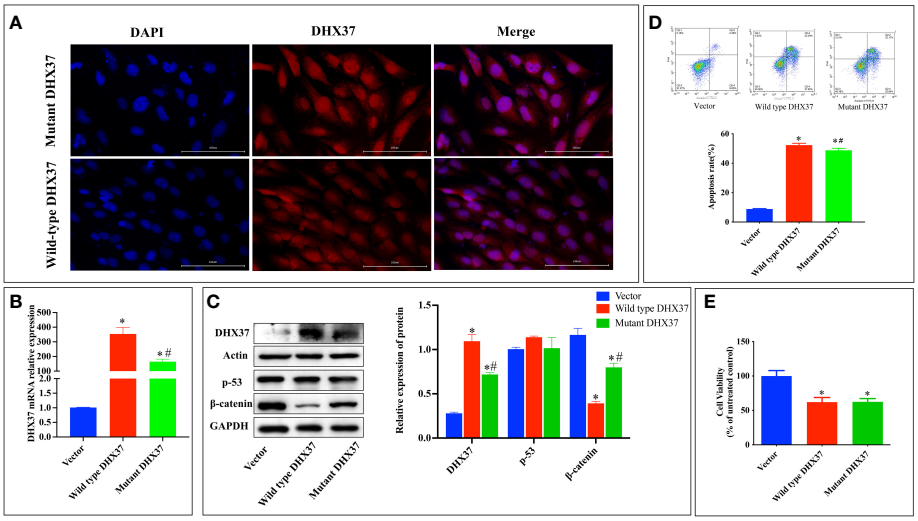

FIGURE 5
In vitro functional studies. **(A)** Cellular localization of mutant and wild-type DHX37 protein. Human Sertoli cells were transfected with wild-type *DHX37* or mutant *DHX37*. Immunofluorescence was performed on the cells using antibodies against DHX37 (red), and the nucleus was stained by DAPI (blue). Scale bars are 100 μm. The highest concentration of the protein was in the nucleus, although protein was also observed in the cytoplasm. Both wild-type and mutant DHX37 exhibited the same cellular localization. **(B)** Relative mRNA expression of *DHX37* when human Sertoli cells were transfected with empty expressing vector, wild-type *DHX37*, and mutant *DHX37*. **(C)** Western blot analysis of DHX37, p53, and the β-catenin protein expression in human Sertoli cells transfected with empty, wild-type, or mutant *DHX37* expression vector. Actin and GAPDH were the housekeeping protein used in this Western blot as a loading control. Effects of the *DHX37* mutation (S408L) on **(D)** apoptosis and **(E)** proliferation of human Sertoli cells transfected with empty, wild-type, or mutant *DHX37* expression vector. Statistical analysis was performed with the use of one-way ANOVA followed by multiple comparisons using a *post hoc* Tukey's test. *p < 0.05 compared with empty expression vector. #p < 0.05 compared with wild-type *DHX37*.

(13, 22). suggested that activation of the canonical WNT/β-catenin pathway may be a possible mechanism. Recent studies revealed that upon nuclear stress challenge, the canonical WNT/β-catenin pathway was transiently activated, followed by p53-dependent apoptosis. It was assumed that activation of the canonical WNT/β-catenin, which regulates a variety of prosurvival processes including cell proliferation and inhibition of cell apoptosis, might serve as a response to sustain nuclear function (20). Notably, inhibition of WNT/β-catenin signaling is necessary for correct testis formation, and stabilization of β-catenin has been identified to cause male-to-female sex reversal in XY gonads (23). With the use of the Sertoli cell line, we demonstrated that overexpression of wt-*DHX37* decreased the expression of the β-catenin protein, which was consistent with the role of DHX37 in the correct formation of testis. However, in the cells transfected with mutant *DHX37* (p.Ser408Leu), the expression of the β-catenin protein was rescued, which may underly the pathogenesis of testicular dysplasia in the current study. Surprisingly, we did not see increases in the p53 protein and cell apoptosis by *DHX37* mutation, which was not supportive of impaired nuclear integrity, raising the possibility that specific biological roles independent of the ribosome formation of DHX37 were involved in the testis development. Future studies may focus on the interaction between DHX37 and β-catenin.

Excision of intra-abdominal gonads is recommended for all XY GD patients, as the risk of germ cell malignancy is as high as 15% ~ 35% (1, 24). Hormone treatment is needed for the induction of puberty, hormone replacement therapy, and suppression of puberty on some occasions based on male/female sex assignment. Overall, in the treatment of DSD, a skilled multidisciplinary group should be involved to facilitate team decisions about assignment/reassignment of male or female sex, surgical issues regarding timing and consent, hormone treatment, and the best possible fertility preservation measures (25). The male patient (III2) was referred to the Pediatric Surgery Department in our hospital for a laparoscopy, and the intra-abdominal gonad bands were removed. Hormone treatment was suggested when it was time for the induction of puberty, usually at age 11–13 in males (25). Surgical exploration was suggested for the proband, since no gonad was found by ultrasound (US). It has been reported that imaging of the gonads by US or MRI is difficult because of the small size and the variable localization in female 46,XY patients (26). Therefore, invasive monitoring is necessary for these patients (18). Unfortunately, after open communication with the patient and her parent, they refused further examination and operation. The patient was upset about her gender identity; therefore, intensive psychological counselling was suggested. If the patient and her parents chose to maintain the female social sex, a low dose of estrogen (one-sixth to one-fourth of an adult dose) was recommended to avoid excessive bone maturation, especially as the patient was concerned about her short stature. Estrogen replacement should be titrated every six months until breast development is complete, after which an adult dose can be maintained continuously (27). Progesterone was not needed in this case, since the patient was found without a uterus (27). However, if the patient chose to change her sex to male, which happens in about 20% of 46, XY DSD patients at a median age of 15 years (28), androgen replacement would be required for masculine pubertal induction.

5 Conclusion

We identified a novel mutant of *DHX37* (c.1223C>T, p. Ser408Leu) in a Chinese pedigree affected by DSD. Bioinformatics analysis suggests the variant is pathogenic, consistent with the *in vitro* study that shows the mutation leads to decreased DHX37 expression both at the mRNA and protein levels. Importantly, in our study, the mutant *DHX37* increases the β-catenin protein, which may be responsible for the disturbance of testis development. Our findings extend the variants associated with DSD and increase the phenotype spectrum associated with DHX37. We also highlight the early diagnosis of 46, XY GD with the use of genetic analysis regarding the high risk of developing gonadal tumors, especially in 46, XY GD females. A definitive genetic diagnosis would be beneficial for screening family members and identifying patients with atypical clinical features, along with prenatal genetic counselling for parents preparing to start a family.

Data availability statement

The sequencing data presented in the study are deposited in the GenBank, accession number OP599354.

Ethics statement

The Ethics Committee approved this study including chromosomal and molecular biology analyses (Institution Review Board of Guizhou Provincial People's Hospital [2021(No. 3)]. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor (s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

KH, LS, and YW designed this project. KH, YW, RY, JL, PH, and XZ participated in the clinical management and data collection. YW and KH organized the genetic analysis. YW, RY, PH, XZ, LS, and KH prepared the manuscript. KH and LS supervised the study and worked on the editing. All authors have read and approved the published version of the manuscript.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1059159/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Karyotype of III:3 **(A)**, III:4 **(B)**, and III:5 **(C)**.

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Insulinoma in childhood: a retrospective review of 22 patients from one referral centre

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Background: Insulinomas are very rare in childhood with sparse knowledge on the clinical aspects and the presence of Multiple Endocrine Neoplasia type 1 (MEN1).

Methods: We conducted a retrospective review of patients diagnosed with insulinoma between 1995 and 2021, presenting to one referral centre in Russia. Clinical, biochemical, genetic, imaging and histological data were collected. In addition, follow-up and family data were obtained.

Results: A total of twenty-two children aged 5 to 16 years were identified. The median (range) gap between the first hypoglycaemia symptoms and diagnosis was 10 (1–46) months. Twelve children (55%) were misdiagnosed to have epilepsy and were treated with anticonvulsants before hypoglycemia was revealed. Contrast enhanced MRI and/or CT were accurate to localize the lesion in 82% (n=18). Five patients (23%) had multiple pancreatic lesions. All children underwent surgical treatment. The median (range) diameter of removed tumors was 1.5 (0.3-6) cm. Histopathological studies confirmed the presence of insulinoma in all cases. Immunohistochemical studies revealed G2 differentiation grade in 10 out of 17 cases. Two patients were diagnosed with metastatic insulinoma. One of them had metastases at the time of insulinoma diagnosis, while the other was diagnosed with liver metastases eight years after the surgery. Eight children (36%) were found to carry *MEN1* mutations, inherited n=5, *de novo* n=1, no data, n=2. Children with MEN1 had significantly higher number of pancreatic tumors compared to sporadic cases. All of them developed additional MEN1 symptoms during the following 2-13

years. In the five patients with inherited MEN1, seven family members had hitherto undiscovered MEN1 manifestations.

Conclusions: In this large cohort of children with rare pediatric insulinomas, MEN1 syndrome and G2 tumors were frequent, as well as hitherto undiscovered MEN1 manifestations in family members. Our data emphasize the need of genetic testing in all children with insulinoma and their relatives, even in the absence of any other features, as well as the importance of a prolonged follow-up observation.

KEYWORDS

insulinoma, hyperinsulinemic hypoglycemia, Multiple Endocrine Neoplasia type 1 (MEN1), pancreatic NETs, malignant insulinoma

Background

Insulinomas are the most common functioning neuroendocrine tumors of the pancreas (pNET), although rare with an incidence of only 1-4 per million per year (1). An incidence peak is in the fifth decade, and insulinomas occur slightly more frequent in women (60%) (1, 2). In the pediatric population, insulinomas are even more rare. Most of the reports in the literature describe single pediatric clinical cases (3–11). There are only few pediatric cohorts of 9-10 cases reported (12, 13).

Insulinomas are usually well-differentiated benign tumors in the pancreas, but malignancy may occur in 5-10% (1). The majority of insulinoma cases are sporadic with only 5-10% of insulinomas being linked with genetic syndromes, of which Multiple Endocrine Neoplasia type 1 (MEN1) is the most common (14). Very few reports in the literature describe the association of insulinoma with neurofibromatosis 1 or tuberous sclerosis (14–16).

Clinically, insulinoma is characterized by recurrent episodes of hypoglycemia. Symptoms typically present after fasting or exercise, but may also develop postprandially (17–19). Biochemical diagnosis corresponds to the criteria of hyperinsulinemic hypoglycemia (HH) and can be established by the presence of detectable serum insulin and C-peptide levels (≥2 U/l and ≥ 0.6 ng/mL, respectively) taken during a hypoglycemic episode with glucose < 3.0 mmol/L. Recurrent hypoketotic hypoglycemia may lead to brain injury, especially in younger age.

In this single-center study, we describe an exceptionally large group of pediatric patients with insulinoma over a 26-year period.

Materials and methods

Study design

A retrospective review of the medical records of pediatric patients (age 0-18 years) diagnosed with insulinoma was performed. Insulinoma was diagnosed biochemically (serum insulin >2.0 U/l during the hypoglycemia <3.0 mmol/l) and by imaging (US, CT, MRI, endoscopic US), and verified by histopathology.

Collected data for the analysis included family history (parents were interviewed on known malignancies, benign lesions, ulcer, cholelithiasis or hypoglycemia in relatives), clinical symptoms prior to diagnosis, its onset and severity, results of the biochemical, hormonal, genetic and histopathological investigations. Whenever possible, clinical data during follow-up period were analyzed.

Screening for the signs of MEN1 syndrome included hormonal analysis (parathyroid hormone (PTH), cortisol, adrenocorticotropic hormone (ACTH), gastrin, prolactin, insulin growth factor 1 (IGF1)), imaging (brain MRI, abdomen US/CT, thyroid US), blood biochemical analysis (Ca, Ca++, glucose, ALT, AST), NET markers (serum chromogranin A and serotonin, urinal 5-Hydroxyindolacetic acid (5-HIAA)) and was performed at the first visit and during follow-up.

Fasting test

Fasting test was performed according to the local protocol and required capillary glucose measurements using an automatic blood glucose meter for professional use every 3 hours if blood glucose (BG) was ≥4 mmol/l, every hour if BG was 3.5-3.9, and every 30 min. if BG was ≤3.4 mmol/l. A critical sample was obtained when BG was less than 3 mmol/L and included serum glucose, insulin and cortisol in all cases. Additionally, serum 3-hydroxybutyrate (BHB), and C-peptide, were measured in 15, and 13 cases, respectively.

Biochemical and hormonal studies

Blood biochemistry was performed using Hitachi 912 Analyzer with standard reagents. Glucose was measured on plasma from venous blood samples with Cobas 8000 hexokinase assay analyzer (Roche®) with normal values 3.3 to 6.1 mmol/l. Bedside glucose values, or continuous glucose monitoring values, were not used for diagnostic fasting measurements. Serum BHB measurements were performed

using a precision Xtra meter (Abbott Pharmaceuticals), with a reported assay range of 0 to more than 8 mmol/L. Urine ketone bodies were measured on an automated iChemVELOCITY analyser (Beckman Coulter Life Sciences, Krefeld, Germany) with urine test strips. Levels of insulin, C-peptide, cortisol, ACTH and PTH were measured using Cobas 6000 analyzer (Roche Diagnostic, Switzerland). Prolactin and IGF1 levels were measured using Vitros 3600 (Johnson & Johnson) and a Liason (DiaSorin) analyzer, respectively. Serum NET markers were evaluated using standard immunoassay method, urine 5-HIAA — using liquid chromatography method.

Histological studies

Histological and immunohistochemical (IHC) studies were performed on sections 3–5 μm thick prepared from paraffin blocks. For IHC studies, an Autostainer (Autostainer, LabVision, type 480s, UK) was used. Sections were deparaffinized and antigenicity was restored in buffer pH 9.0 in a PT Module (Thermo Scientific, UK). To determine the type of tumor, a spectrum of antibodies was used: Chromogranin A (clone LK2H10), synaptophysin (clone MRQ-40), CD56 (clone MRG42), insulin (polyclone, RTU), glucagon (polyclone, RTU), somatostatin (polyclone, RTU, Cell Marque, USA), and gastrin (all from Cell Marque, USA), pancreatic polypeptide (clone EPR2330-10, Abcam, USA), Ki67 (clone MIB1, DAKO), somatostatin receptors type 2 (rabbit monoclonal EP149, Epitomix, USA) and type 5 (rabbit monoclonal UMB4, Epitomix, USA).

Somatostatin receptor (SSTR) expression analysis was performed according to the method of Volante M. et al.(20). Membrane expression of SSTR2, or membrane-cytoplasmic expression of SSTR5, was considered as positive if found in more than 30% of tumor cells.

The expression of other cytoplasmic markers was estimated according to standard semi-quantitative method for cytoplasmic markers. Tumor grade was assessed using Ki67-index according to the World Health Organization guideline (21).

Genetic studies

Genomic DNA from peripheral blood leukocytes was extracted using standard methods (22). Molecular genetic analysis of *MEN1* was performed using bidirectional direct sequencing (n=21). Samples were sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, FosterCity, CA, USA) and analyzed on a ABI3730XL DNA Analyzer (AppliedBiosystems, Naerum, Denmark). Sequence analysis was performed using SeqMan Software (DNASTAR, Madison, WI, USA). To detect larger deletions Multiplex Ligation-dependent Probe Amplification (MLPA) analysis was performed (n=4) according to the manufacturer's recommendations (Salsa MLPA, P017 MEN1, MRC-Holland, Amsterdam, the Netherlands). Data were analyzed using GeneMarker (Softgenetics, Pennsylvania, USA). *MEN1* DNA variant nomenclatures were given according to GenBank accession no. NM_000244.4.

Follow-up studies

Follow-up investigations included screening for MEN1 components as described for the first visit, including detection of any distant metastases, using abdominal ultrasound or MRI. In patients with genetically verified MEN1 syndrome first grade relatives (parents and siblings) underwent genetic testing for point mutation in *MEN1* gene and in case of positive results, — screening for MEN1 components as mentioned above.

Statistics

Demographic and clinical data were presented as median (interquartile range (IQR)). Statistical analysis was performed using StatSoft Inc., USA, version 10.0. Non-binary data were analyzed with the help of Mann-Whitney U test and chi-square test with p value < 0.05 considered as significant.

Results

Patient demographics and characteristics

We analyzed 465 medical records of children aged less than 18 years admitted to the Endocrine Research Center with HH from 1995 to 2021. A total of 22 patients (13 females) were diagnosed with primary pancreatic insulinoma, accounting for 4.7% of all pediatric patients with HH.

The median age at the time of first symptoms was 10.45 years. Median age at the time of diagnosis was 11.5 years, giving the median delay in diagnosis of 10 months (Table 1). Twelve children (55%) were misdiagnosed to have epilepsy and were treated with anticonvulsants before the hypoglycemia was revealed. All patients had typical clinical features of hypoglycemia, including drowsiness (73%), seizures (73%), syncope (68%), progressive weight gain (45%), learning and behavioral difficulties (45%). A third of the patients experienced hypoglycemic coma prior to diagnosis (7/22; 32%).

Hypoglycemia evaluation

Median duration of diagnostic fasting test was eight hours (Table 1). In all cases it resulted in laboratory hypoglycemia with a mean serum glucose level of 1.76 ± 0.63 mmol/L. Median serum insulin level taken during hypoglycemia was 20.9 U/L, median C-peptide level 3.4 ng/mL (n=13). Serum hydroxybutate was less than 0.5 mmol/l in all cases (n=15). Urine ketone bodies were undetectable in all measurements in cases when serum ketones were not available (n=7).

TABLE 1 Main clinical and biochemical parameters of 22 pediatric patients with insulinoma.

Characteristics	Results
Male : Female, absolute numbers	9:13
Age at the onset	10.45 (5.1-16.2) years
Age at the time of diagnosis	11.5 (7.7-16.8) years
Fasting test duration*	8 (1–19) hours
Serum glucose level at the end of the fasting test*	1.9 (0.5-2.2) mmol/L
Serum insulin level at the end of the fasting test*	20.9 (8.13-149) U/L
Serum C-peptide level at the end of the fasting test**	3.4 (1.96-10.2) ng/mL

All data are given as median (range) if not otherwise indicated. *Performed in 21/22 cases. **Performed in 13/22 cases.

Insulinoma imaging

Localization of the tumor only by transabdominal ultrasound was possible in seven patients. Contrast enhanced CT and/or MRI were used in 18 cases. Additional endoscopic ultrasound was needed in four cases with inconclusive results of MRI and CT. Figure 1 represents results of different imaging technics used for the evaluation of pancreatic lesions in our cohort of patients.

Five children (23%) had multiple pancreatic lesions. The 30 lesions were found equally in all parts of the pancreas (tail; n=11, head; n=10, body; n=9).

Hypoglycemia management

Prior to surgery, 12 children required hyperglycemic medication. Diazoxide and octreotide alone were used in nine and two cases, respectively. One patient (case#12) had a combination of both drugs. Doses of diazoxide ranged from 100 to 300 mg/day. In 7 out of 10 patients, diazoxide was sufficient to maintain normoglycemia. Octreotide was given by subcutaneous injections every 8 hours in doses of 400 mcg/day. Other patients were managed with frequent feeds and/or continuous dextrose infusion.

Pancreatic surgery

Surgical treatment was performed in all children: insulinoma enucleation in 11 (50%), partial pancreatic resection in 8 (36%), subtotal pancreatectomy in 2 (9%). In one patient (5%) with multiple lesions, repeated surgeries ended up with pancreatogastroduodenal resection (Table 2). Removed tumors varied in size from 0.3 to 6 cm. Six tumors were less than 1 cm, 14 ranged from 1 to 2 cm, and 10 were larger than 2 cm.

Histopathology results

Histopathological studies confirmed the presence of insulinoma in all cases. Lesions showed polymorphic histology: most of the tumors had trabecular architecture (Figures 2D–O), rarely solid (Figures 2A–C) or mixed. Majority of lesions were encapsulated and well circumscribed from the surrounding pancreatic tissue. One patient (case #12) had multifocal insulinoma without capsule and invasion to the surrounding tissue (Figures 2M–O). This patient had liver metastases at the time of diagnosis.

Immunohistochemical studies were performed in 17 cases. All lesions demonstrated the expression of Synaptophysin and Chromogranin A. Among the children with multiple pancreatic lesions (n=5), some of the NETs were negative for insulin staining. Of these, glucagon expressing tumors were found in two cases (Figures 2H–L). Others were classified as non-functioning. Compound expression of gastrin and insulin was found in 1 case. SSTR2 and SSTR5 expression was analyzed in 13 lesions and was positively expressed in 6 and 5 cases, respectively (Figures 2G, K, L).

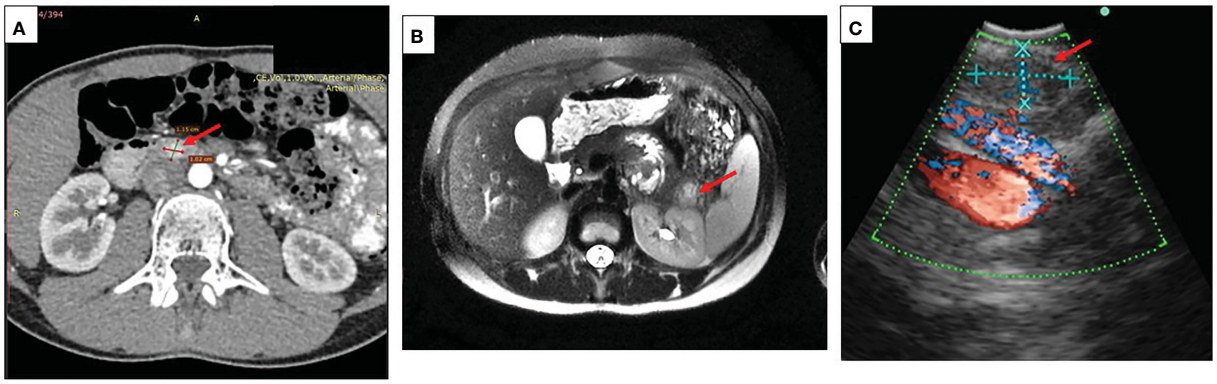

FIGURE 1
Imaging in pediatric patients with insulinoma **(A)** A representative CT scan showing insulinoma in the head of the pancreas (case #14), arrow indicating a round shaped hypervascular 1.5 cm lesion in the head of the pancreas highly enhanced during the arterial phase; **(B)** A representative contrast MRI scan showing an insulinoma in the pancreatic tail (case #8), arrow indicating a 2.3 cm lesion in the pancreatic tail with high signal intensity on T2 MR regimen; **(C)** A representative picture of endoscopic ultrasound localizing lesion in the pancreatic tail (case #13), arrow indicating a 0.9 cm ovoid shaped lesion, isoechoic with hypoechoic inclusions and distinct margins.

TABLE 2 Comprehensive clinical data on 22 pediatric patients with insulinoma and their family members.

Case	MEN1 mutation, Inheritance	Age at onset, years	Age at diagnosis, years	Treatment prior to surgery	Type of surgery	Amount of pNETs (n) and size	Grade	Follow-up duration, years	Follow-up findings and treatment	Family history	Family members' investigation results
1	c.830C>G p.P277R Paternal	9.5	11.3	Frequent feeds	Partial resection	N=2. ⌀ 1.2cm; 0.3cm	G2	4.2	Pituitary adenoma at 13 years; hPTH at 15 years	Peptic ulcer in father	hPTH in father, hPRL in cousin
2	c.1547insC, p.(Lys517Glufs*14) Unknown inheritance	7.3	8.3	Glucose infusion	Subtotal pancreatectomy	N=1. ⌀ 2.5cm	No data	17.5	hPRL at 21 years (on Cabergoline); Gastrinoma at 25 years (surgical treatment).	Gastric cancer in grandmother	No data
3	c.936delC, p.(Tyr313Ilefs*55) Paternal	9.4	13.2	Frequent feeds	Enucleation	N=1. ⌀ 3cm	No data	6.1	hPTH, hPRL, adrenal nodular hyperplasia at 19 years	Unremarkable	hPTH in father and brother
4	c.784-9G>A p.? HGMD no. CS991446 Maternal	8.2	10.9	Frequent feeds	Enucleation	N=2. ⌀ 1.5cm; 1.4cm	G2	5.2	hPTH at 13 years; hPRL at 16 tears (on Cabergoline).	Unremarkable	Unremarkable
5	c.625_628delACAG p.T210Sfs*13 Paternal	5.1	8.8	DZX	Partial resection	N=3. ⌀ 2.0 cm; 0.6 cm; 0.5cm.	G1	5.3	Somatotropinoma at 12 years; pNET at 13 years; hPTH at 14 years. Underwent parathyroidectomy and second pancreatic surgery (previously on SST analogues for 3 years).	Unremarkable	Glucose intolerance, hypercalcemia in father
6	c.923C>A p.S308* HGDM no. CM970932 Unknown inheritance	13.3	13.8	DZX	Enucleation	N=2. ⌀3.7 cm; 0.6 cm	G2	4.5	hPTH at 18 years	Father died at 35 years (reason unknown)	No data
7	c.133G>A p.E45K Paternal	7.1	7.7	DZX	Partial resection	N=1. ⌀ 1.1cm	G2	1	None	Unremarkable	hPTH in father
8	c.141dup p.Leu48Serfs*69 DeNovo	11.4	11.5	Frequent feeds	Enucleation	N=1. ⌀ 2.3cm	G2	0.5	None	Unremarkable	No data
9	Negative	16.2	16.8	Frequent feeds	Enucleation	N=1. ⌀3.5cm	G2	1.5	None	Colon cancer in grandmother; Lung cancer in	No data

(Continued)

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TABLE 2 Continued

Case	MEN1 mutation, Inheritance	Age at onset, years	Age at diagnosis, years	Treatment prior to surgery	Type of surgery	Amount of pNETs (n) and size	Grade	Follow-up duration, years	Follow-up findings and treatment	Family history	Family members' investigation results
										father`s sister at 25 years	
10	Negative	11.2	12.6	Glucose infusion	Enucleation	N=1. ⌀ 3.0cm	G1	2.2	None	Colorectal cancer in mother`s brother at 42 years	No data
11	Negative	10.8	11.5	Frequent feeds	Enucleation	N=1. ⌀ 1.5cm	G2	2.1	None	Unremarkable	No data
12	Not done	11	11.1	Octreotide + DZX + glucose infusion	Subtotal pancreatectomy + splenectomy	N=1 ⌀ 6cm + Multiple liver mts	G2 in tumor G3 in mts	0.5	Deceased at 11 years 8 months. Previously treated with SST + mTOR inhibitors.	Unremarkable	No data
13	Negative	7.3	7.7	DZX	Enucleation	N=1. ⌀ 0.95cm	G1	No data	No data	Unremarkable	No data
14	Negative	12.3	14.5	Octreotide	Pancreatic resection	N=1. ⌀ 1.56cm	G1	No data	No data	Peptic ulcer in mother; Hypoglycemia ()? in maternal cousin	No data
15	Negative	13.5	14.1	DZX	Partial resection	N=1. ⌀ 2cm	G1	1.3	Kreon	Pancreatic cancer in maternal grandmother	No data
16	Negative	12.4	13	Octreotide	Partial resection	N=1. ⌀ 1.5cm	G2	0.5	None	Gastric cancer in maternal sister	No data
17	Negative	16.1	16.3	DZX	Partial resection	N=1. ⌀ 1.1cm	G1	No data	No data	Gastric and colon cancer in paternal grandfather	No data
18	Negative	10.1	13.8	Frequent feeds	Enucleation	N=1. ⌀ 1.9cm	No data	5.1	Epilepsy at 18 tears	Unremarkable	No data
19	Negative	7.3	11	Frequent feeds	Enucleation	N=1. ⌀ 1cm	No data	4.5	None	Rectal cancer in grandfather	No data
20	Negative	10.1	11.2	DZX	Enucleation at 11 years; Partial resection at 12 years; Pancreato-gastro-	N=4. ⌀ 1.5cm; 0.5cm; 1.0cm; 3.0cm.	G1-G2	21.5	Postoperative DM; Liver mts at 21 years; Nephropathy at 32 years. Treated with SST analogues, mTOR inhibitors, dialysis.		No data

(Continued)

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TABLE 2 Continued

Case	MEN1 mutation, Inheritance	Age at onset, years	Age at diagnosis, years	Treatment prior to surgery	Type of surgery	Amount of pNETs (n) and size	Grade	Follow-up duration, years	Follow-up findings and treatment	Family history	Family members' investigation results
					duodenal resection at 13 years						
21	Negative	8.0	9.1	DZX	Enucleation	N=1. ø 1.9 cm	G1	No data	No data	No data	No data
22	Negative	15.8	16.5	DZX + glucose infusion	Partial resection	N=1. ø 2.5 cm	No data	No data	No data	No data	No data

DZX, diazoxide; pNET, pancreatic neuroendocrine tumor; N, number of pNETs; ø, diameter; nts, metastasis; G1, low tumor grade; G2, intermediate tumor grade; SST, somatostatin; hPTH, hyperparathyroidism; hPRL, hyperprolactinemia; HGMD, Human Gene Mutation Database; ®, mTOR inhibitors, mammalian target of rapamycin inhibitors; DM,m diabetes mellitus.

Ki67 index and/or mitotic indexes were measured in a total of 19 lesions. In ten cases we found G2 differentiation grade (Table 3).

MEN1 evaluation

We performed biochemical screening for the MEN1 syndrome components at the time of insulinoma diagnosis. This revealed mild normocalcemic hyperparathyroidism in two patients (cases #4 and #5), mildly elevated serotonin in three patients (cases #4,5,6), and high levels of Chromogranin A and 5-HIAA in one (case #6). There were no biochemical or radiological signs of pituitary adenoma. Analysis of the family history was possible in 20 cases and revealed malignancies in relatives (n=6), ulcer (n=2) and hypoglycemia (n=1). None of the interviewed relatives had a history of hyperparathyroidism or insulinoma (Table 2).

MEN1 gene sequencing revealed pathogenic variants in 8 out of 21 children (38%). Of them, 2 missense, 1 nonsense, 1 splicing and 4 frameshift mutations were identified (Table 2). Five variants were previously described in MEN1 patients (23–27). Mutations p.P277R (case #1), p.Tyr313Ilefs*55 (case #3), and p.Leu48SerfsTer69 (case #8) are novel. With regards to a latter variant, a different substitution in the same codon was previously described in the literature as disease causing (28).

We compared main clinical characteristics in children with sporadic insulinomas (n=13) and those with pathogenic variants in the *MEN1* gene (n=8) (Table 4). Children with MEN1 syndrome tended to be younger at the time of insulinoma presentation, with a higher number of lesions and more often G2 grade. Significant difference between the two groups was, however, only found in the number of lesions (Table 4).

Genetic testing of parents was performed in six families and revealed MEN1 carriers in five. All relatives with *MEN1* mutations were investigated. Despite the absence of suspicious family history and minimal clinical presentations, four of out the five *MEN1* parental carriers and two additional relatives were found to have components of MEN1 syndrome, but none compatible with insulinoma (Table 2).

Follow-up data were available in 17 patients. Median [25-75%] follow-up duration was 4.2 [1.3-5.2] years. There was no recurrence of insulinoma during follow-up. All children with genetically confirmed MEN1 syndrome developed various MEN1 components during next 2-13 years with hyperparathyroidism and hyperprolactinemia being the most common findings (n=5 and 3 resp.), (Table 2). In one patient, liver metastases were found eight years after the pancreatectomy (case #20).

Discussion

While congenital hyperinsulinism is the most common cause for persistent and recurrent hypoglycemia in infancy (29), the possibility of insulinoma should be considered in those with HH presenting after the age of three years. In our group, the youngest age at onset was five years. Literature reports describe cases of insulinoma in even younger children (12). As

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well as others, we noticed a pronounced delay in diagnosis, which was approximately a year since the first symptoms of hypoglycemia (12). The delay can be explained by the extreme rarity of the condition and the nonspecific, episodic symptoms. Most of the children in our group experienced hypoglycemia only after prolonged fast. In contrast to adults, children with insulinoma tend to develop neuroglycopenic symptoms of hypoglycemia more often (3). In our cohort, 73% of patients had hypoglycemic seizures that led to a misdiagnosis of epilepsy in a half of cases.

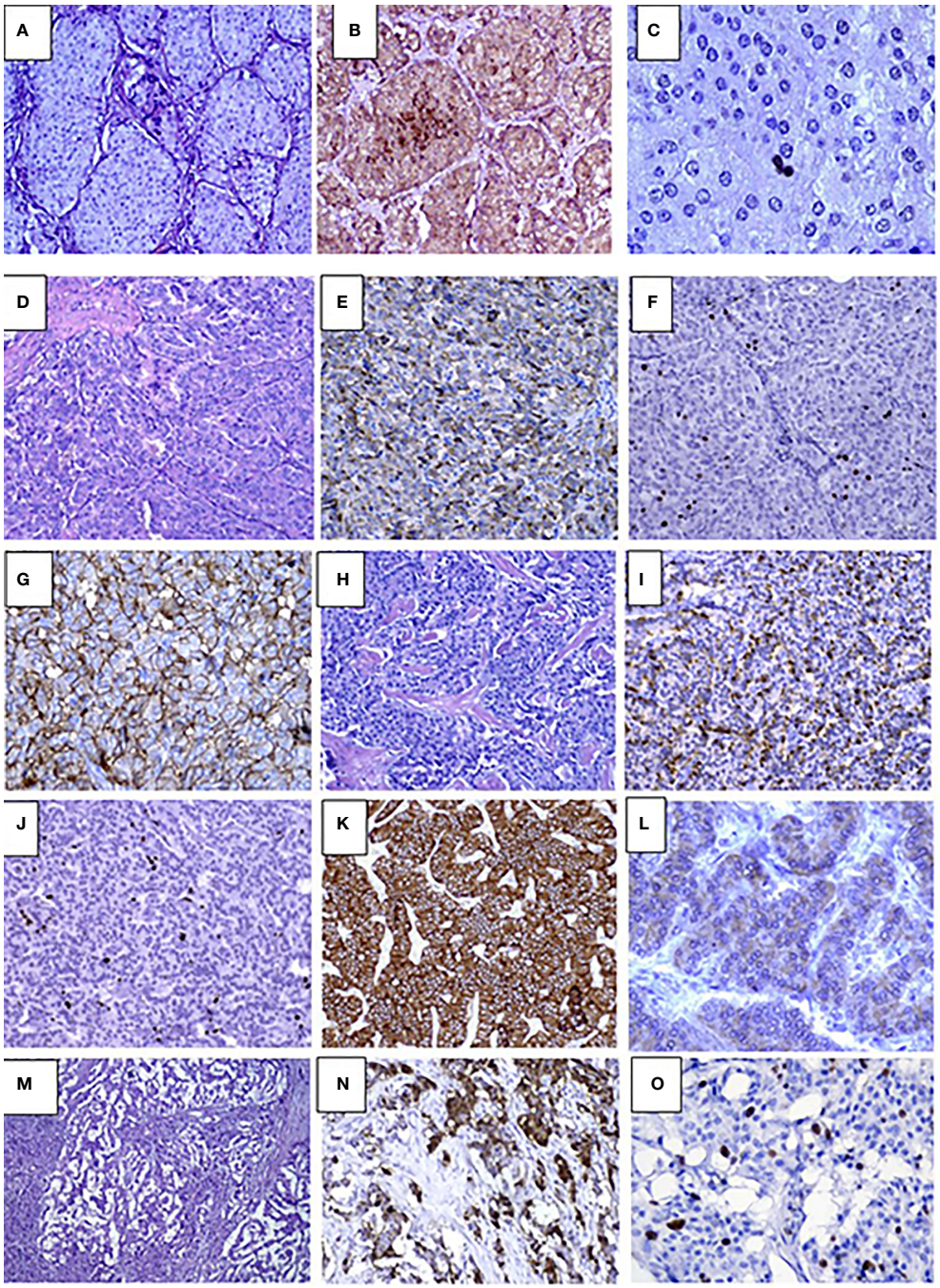

FIGURE 2
Pathology results in pediatric patients with insulinoma **(A–C)**. Case #10. Solid G1 insulinoma composed of packets of monomorphic cells, H&E, x250 **(A)**, Immunohistochemistry staining with insulin shows abundance of insulin secreting cells within the lesion, x250 **(B)**, Ki67 positive staining in single nuclei of tumor cells (1%) x400 **(C)**. D-L. Case #4. Trabecular G2 insulinoma with poorly developed stroma, H&E, x250 **(D)**, insulin staining, x250 **(E)**, Ki67 staining x250 **(F)**, membrane expression of SSTR type 2 in tumor cells (3+), x250 **(G)**; trabecular glucagon positive and insulin negative NET, G2, H&E, x250 **(H)**, moderate apical glucagon expression in tumor cells, glucagon staining x 250 **(I)**, Ki67 staining, x250 **(J)**, abundant membrane expression of SSTR type 2 (3+), x250 **(K)**, mild expression of SSTR type 5 (2+), x400 **(L)**, M-O. Case #12. Multifocal invasive insulinoma with trabecular architecture G2, H&E, x250 **(M)**, large complexes and small tumor cell nests expressing insulin, Insulin staining, x250 **(N)**, intensive Ki67 expression in cell nuclei, Ki67 staining, x400 **(O)**.

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TABLE 3 Pathology results for 22 pediatric patients with resected insulinoma.

Case	n of lesions	Max Size in Diameter (cm)	localization	Morphology features	Immunohistochemistry							
					Syn	CgA	Ins	Gastr	Gluc	SSTR2/ SSTR5	Ki67 (%)	Grade
1	2	1.2	Head	solid	+	+	+	-	-	ND	3.5	G2
		0.3	Tail	solid	+	+	+	-	-	ND	ND	ND
2	1	2.5	Head	ND	ND	ND	ND	ND	ND	ND	ND	ND
3	1	3	Tail	mixed	ND	ND	ND	ND	ND	ND	ND	ND
4	2	1.5	Body	solid	+	+	+	-	-	+/+	11	G2
		1.4	Tail	trabecular	+	+	-	-	+	+/+	12	G2
5	3	2	Body	mixed	+	+	+	-	-	-/+	1	G1
		0.6	Tail	solid	+	+	+	-	-	-/-	1	G1
		0.5	Head	solid	+	+	-	-	-	-/-	1	G1
6	2	3.7	Head	Solid	+	+	+	-	-	+/+	11	G2
		0.6	Tail	trabecular	+	+	-	-	+	-/-	8	G2
7	1	1.1	Tail	mixed	+	+	+	ND	ND	-/-	2	G2
8	1	2.3	Tail	trabecular	+	+	+	-	-	-/-	10.5	G2
9	1	3.5	Tail	mixed	+	+	+	-	-	+/-	10.5	G2
10	1	3.0	Tail	solid	+	+	+	-	-	ND	1	G1
11	1	1.5	Body	trabecular	+	+	+	+	-	+/-	4	G2
12	1	6	Body	trabecular	+	+	+	-	-	ND	8% in the tumor, 21.5% in mts	G2 in tumor, G3 in Mts
13	1	0.95	Tail	mixed	ND	ND	+	ND	ND	ND	1	G1
14	1	1.56	Head	mixed	+	+	+	-	-	ND	1	G1
15	1	2	Head	trabecular	+	+	+	-	-	ND	1	G1
16	1	1.5	Head	mixed	+	+	+	ND	ND	ND	4	G2
17	1	1.1	Tail	trabecular	+	+	+	-	-	+/+	1	G1
18	1	1.9	Head	ND	ND	ND	ND	ND	ND	ND	ND	ND
19	1	1	Body	solid	ND	ND	+	ND	ND	ND	ND	ND
20	4	1.5	Head	trabecular	ND	ND	+	ND	ND	ND	ND	G1
		0.5	Head	ND	ND	ND	ND	ND	ND	ND	ND	ND
		1	Body	ND	ND	ND	ND	ND	ND	ND	ND	ND
		3	Body	mixed	ND	ND	ND	ND	ND	ND	ND	G2
21	1	1.9	Body	trabecular	+	+	+	-	-	-/-	1	G1
22	1	2.5	Body	mixed	+	+	+	ND	ND	ND	ND	ND

ND, no data; SYN, synaptophysin; CgA, chromogranin A; Ins, insulin; Gastr, gastrin; Gluc, glucagon; SST2, somatostatin receptors type 2; SST5, somatostatin receptors type 5; mts - metastases. "+", positive staining; "-", - negative stainingm.

According to a recent review, routine imaging techniques such as transabdominal ultrasound, CT and MRI have relatively low accuracy for insulinoma localization with estimated sensitivity of 9-66%, 35-82% and 35-63%, respectively (30). Endoscopic ultrasound seems to be the most accurate diagnostic tool for insulinomas with a sensitivity of 94% alone or up to 100% if combined with CT scan (31). Recently invented imaging technics such as 68Ga-DOTATATE PET and GLP-1 receptor scintigraphy are widely used in patients where the first-line imaging tests are unable to detect the lesion (32).

TABLE 4 Comparative analysis of the clinical features in pediatric patients with sporadic vs. MEN1-associated insulinoma.

	Sporadic insulinoma n=13	MEN1 syndrome n=8	p
Male : Female ratio	5:8	3:5	N.S.
Age at onset, years Median [Q25-Q75]	11.2 [10.1-13.5]	9.3 [7.2-9.45]	N.S.
Multiple pancreatic lesions (patient n, %)	1/13 (7.7%)	4/8 (50%)	p=0.028
Tumor size, cm Median [Q25-Q75]	1.53 [1.07-2.12]*	1.4 [0.6-2.3]**	N.S.
G2 grade n/n (%)	4/10 (40%)	5/6 (83.3%)	N.S.
Serum insulin (U/L) during hypoglycemia Median [Q25-Q75]	21.84 [14.4-41.1]	15.9 [12.1-30.4]	N.S.

N.S., Non significant. *n=16, **n=13.

In our cohort, routine imaging technics were accurate in 86% of cases. This finding fits with previous publications on pediatric insulinomas where MRI alone localized pancreatic lesions in 88% (7/8) of patients (12). The higher imaging sensitivity compared to adults may be related to a bigger tumor size in the pediatric cohorts. For instance, in our group lesions were ≥1 cm in diameter in 80% and ≥ 2 cm in 33% of cases, whereas in adults insulinomas usually do not exceed 1 cm (33). We speculate that insulinomas in childhood grow more rapidly, rather than having a longer diagnostic delay, compared to insulinomas in adults.

There is an association between size of the tumor and its malignancy potential (34). Malignancy of the insulinoma is only defined by the presence of metastases or the invasion in surrounding organs (35). Malignant insulinoma is rare and accounts for only 5-10% of all cases of insulinoma (36) with only few reports of pediatric cases in the literature (4, 7, 37). Insulinomas are usually classified using the 2017 WHO grading system which is mainly based on mitotic and/or Ki67 index (21), although a new classification has recently been suggested (38). According to the latter insulinomas can be divided into two subtypes: "Typical" insulinomas that have strong epigenetic similarities to pancreatic beta-cells (*PDX1*-positive/*ARX*-negative) and a favorable prognosis after the complete surgical resection. These typical insulinomas become symptomatic very early when they are small in size (< 2 cm) and are characterized by somatic *YY1* mutations in about 30% of cases, or recurrent somatic amplifications (in particular chromosome 7 amplifications) (39).

Another subtype consists of rare clinically aggressive "atypical" insulinomas. They do express *ARX* and are characterized by large tumor size (3.5–9 cm) and metastatic behavior (40). *ARX*-positive insulinomas show genetic alterations also seen in non-functioning pNETs, such as loss of *ATRX/DAXX* and *CDKN2A*. It has been suggested that atypical insulinomas most likely exist as non-functioning pNETs for a time before becoming clinically functioning (41).

We did not perform genetic studies of tumor cells, but may suspect that one of our patients (case#12) had an "atypical" insulinoma presenting with multiple liver metastases at the time of diagnosis.

The other patient with high malignancy-potential insulinoma in our cohort (case #20; no MEN1 mutations) presented with multiple pancreatic lesions, requiring pancreatic gastroduodenal resection, but distant metastases were found eight years after the surgery. According to the literature, patients may develop metastatic disease several years after excision of insulinomas that initially were considered benign. This relapse risk is more probable in grade G2 tumors (36, 42). In our cohort, G2 differentiation grade of the tumor was found in 60% of cases. These data, together with our observation of distant metastases found almost a decade after the pancreatic gastroduodenal resection, urge for long and specific follow-up of all children with insulinoma, with or without MEN1.

Little is known on the efficacy of hyperglycemic drugs in children with insulinomas. In adults with insulinoma, diazoxide was shown to be effective in approximately 40-60% (43). According to a recent publication, children with insulinomas are less responsive to diazoxide therapy (12). In our cohort, only 10 out of 22 children received diazoxide, of which seven showed some response to it, although we do not have data on control fasting tests. Since the presence of somatostatin receptors was observed in insulinomas, treatment with somatostatin analogs has also been used in insulinoma patients (44). However, the usefulness of somatostatin analogs in the treatment of insulinoma patients remains controversial (45). In our group, somatostatin analogues were used in three patients only and did not significantly improve glycemia. Unfortunately, we lack the data on somatostatin receptors expression in these cases.

MEN1 syndrome is known to be responsible for about 4-7% of insulinoma cases in adults (1), whereas our pediatric cohort had a much higher frequency of 38%. Interestingly, all of our patents had insulinoma as the first MEN1 manifestation, while other components of the syndrome developed later in life. When comparing the main clinical and histological features of sporadic and MEN1 patients in our cohort, a tendency towards earlier onset and higher proliferative index of the lesions, as well as significantly higher number of pNETs among MEN1 cases were observed. Multiple pancreatic lesions among sporadic cases were found in one patient only (case#20), which probably represent a metastatic invasion of the pancreas rather than primary multiple tumors.

We did not observe a relapse of the insulin producing tumors during the follow-up period in sporadic or MEN1 patients. In one of the MEN1 patients (case# 5), additional pNETs were found during follow-up, leading to a second surgery at the age of 16 years where three tumors were removed. Both preoperative blood biochemical tests and postoperative immunohistochemical studies confirmed the absence of insulin secreting cells in these lesions.

Apart from the MEN1 gene, only few genes are known to cause insulinoma (14, 16). Very few reports in the literature describe the association of insulinoma with neurofibromatosis 1 and tuberous sclerosis (3–5). In our cohort, children did not present with any specific clinical features but hypoglycemia and, therefore, were tested for the MEN1 mutations only. Several studies have been performed to evaluate the possible genetic background of the tumorigenesis of insulinoma and multiple candidate genes have been identified (46). In pediatric insulinomas, aneuploidy of chromosome 11 and other chromosomes have been found to be common in both MEN1 and non-MEN1 patients (13). Further experiments are essential to validate the clinical relevance of these findings.

Limitations of this study includes its retrospective design and missing data on family history and patient's current state. Some of the family members declined to undergo the investigations, or were not available for interview. Another limitation is the risk of type 2 statistical errors due to the extreme rareness of the disease in the pediatric population. Strengths of the study include, on the other hand, the unique high number of patients included, the detailed clinical and paraclinical work-up and the relatively long follow-up time.

Conclusion

In this exceptionally large cohort of rare pediatric patients with primary insulinomas, we identified a high incidence of MEN1 syndrome. There was no significant difference in clinical features of sporadic and MEN1 cases, emphasizing the need for genetic testing in all children with insulinoma even in the absence of any other features. Review of the pathology results showed a high prevalence of G2 tumors in our patients. Even though malignant insulinomas are extremely rare in young children, we described the possibility of distant metastases developing many years after the diagnosis, indicating the importance of a prolonged follow up of the patients.

Data availability statement

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

Ethics statement

This study was performed in line with the principles of the Declaration of Helsinki. The studies involving human participants were reviewed and approved by the local ethics committee of The Endocrinology Research Center (protocol number 10 from 26/05/2021). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin prior to genetic testing which was conducted as part of routine clinical care.

Author contributions

MM, DG contributed in conception and design of the study and writing the manuscript, ASh wrote the first draft of the manuscript, AB, MK, AE, YS, SM, JA and ASt collecting data, KB and AT collecting of genetic results and analysis, HTC and KA major revision of the manuscript, LG - collecting the pathology data and analysis, major revision of the manuscript. All authors contributed to manuscript revision, read, 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.

Publisher's note

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Paediatric Wolfram syndrome Type 1: should gonadal dysfunction be part of the diagnostic criteria?

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Aims: Wolfram Syndrome Spectrum Disorder (WFS1-SD), in its "classic" form, is a rare autosomal recessive disease with poor prognosis and wide phenotypic spectrum. Insulin dependent diabetes mellitus (DM), optic atrophy (OA) diabetes insipidus (DI) and sensorineural deafness (D) are the main features of WFS1-SD. Gonadal dysfunction (GD) has been described mainly in adults with variable prevalence and referred to as a minor clinical feature. This is the first case series investigating gonadal function in a small cohort of paediatric patients affected by WFS1-SD.

Methods: Gonadal function was investigated in eight patients (3 male and 5 female) between 3 and 16 years of age. Seven patients have been diagnosed with classic WFS1-SD and one with non-classic WFS1-SD. Gonadotropin and sex hormone levels were monitored, as well as markers of gonadal reserve (inhibin-B and anti-Mullerian hormone). Pubertal progression was assessed according to Tanner staging.

Results: Primary hypogonadism was diagnosed in 50% of patients (n=4), more specifically 67% (n=2) of males and 40% of females (n=2). Pubertal delay was observed in one female patient. These data confirm that gonadal dysfunction may be a frequent and underdiagnosed clinical feature in WFS1-SD.

Conclusions: GD may represent a frequent and earlier than previously described feature in WFS1-SD with repercussions on morbidity and quality of life. Consequently, we suggest that GD should be included amongst clinical diagnostic criteria for WFS1-SD, as has already been proposed for urinary

dysfunction. Considering the heterogeneous and elusive presentation of WFS1-SD, this clinical feature may assist in an earlier diagnosis and timely follow-up and care of treatable associated diseases (i.e. insulin and sex hormone replacement) in these young patients.

KEYWORDS

Wolfram syndrome, Wolfram syndrome 1 (WFS1), monogenic diabetes, gonadal dysfunction, hypogonadism, hypergonadotropic hypogonadism

1 Introduction

Classic Wolfram Syndrome Spectrum Disorder (WFS1-SD) is a rare genetic condition with autosomal recessive transmission and an estimated prevalence of 1:160.000 - 1:770.000 (1, 2). WFS1-SD is caused by mutations in the gene encoding wolframin (WFS1), a protein primarily located in the endoplasmic reticulum (ER) (2). Consequent dysregulation in ER and cytoplasmic Ca2+ homeostasis seems to be the main process leading to apoptosis and degeneration involving β- and neural cells (3–7). Main clinical features of classic WFS1-SD include diabetes mellitus (DM) optic atrophy (OA) diabetes insipidus (DI), and sensorineural deafness (D), hence the acronym DIDMOAD (2, 8). Nonetheless, clinical presentation is extremely variable and often complicated by neurological, urological, psychiatric and other endocrine dysfunctions (2, 4, 8–10). To date, mutations of WFS-1 with dominant transmission have been described (11). Classified as non-classic WFS1-SD, these defects determine deafness (sometimes associated with milder optic atrophy), diabetes alone or congenital cataracts (5, 12, 13).

Currently, there is no treatment able to delay, stop, or reverse the natural history of disease (5). Novel and already marketed/repurposed drugs are being assessed as possible disease modifying agents (2, 5, 7).

Gonadal dysfunction, mainly hypogonadotropic hypogonadism, has been reported and is to date considered a minor clinical feature in WFS1-SD patients (14, 15). Hypergonadotropic hypogonadism has also been recently described (5, 14–18).The prevalence of both primary and secondary hypogonadism in WFS1-SD varies widely from different reports, ranging from 7.1% to almost 30% (16, 19, 20).

To date, gonadal failure has been observed more frequently in young male adults with heterogeneous manifestations. In males, associated features described with hypogonadism included atrophic testes, small penis, erectile dysfunction, gynecomastia and poor secondary sexual characteristics (2, 5, 15, 17, 19, 21). Females may often generally present with primary amenorrhoea (22). Interestingly, the first case of primary hypergonadotropic hypogonadism in a female patient with confirmed WFS1-SD has been recently described in a 16 year adolescent (23). Pubertal delay is often observed in WFS1-SD adolescents alongside growth delay due to hypopituitarism (15, 23). Nevertheless, cases of successful pregnancy and delivery exist and first reports of male fertility date back to a decade ago (15, 24, 25).

The aim of our study was to assess gonadal function in a small cohort of eight paediatric patients (3 male and 7 female) with WFS1-SD undergoing regular follow-up at IRCCS San Raffaele Hospital in Milan.

2 Materials and methods

2.1 Population

Our study group includes eight patients between 3 and 16 years old. Seven patients have been diagnosed with genetically confirmed classic WFS1-SD and one (the youngest female) non-classic WFS1-SD. All patients are currently undergoing an off-label treatment with subcutaneous liraglutide alongside classic treatments required for diseases associated with the syndrome (i.e. insulin therapy, growth hormone replacement therapy). Patients 1 to 4 were previously included in a published case series assessing safety and tolerability of liraglutide in paediatric WS (26)

2.2 Diagnostic assessment

Hypothalamic-pituitary-gonadal axis was assessed by serum gonadotropin (luteinizing hormone, LH and follicle-stimulating hormone, FSH) and sex hormones (testosterone in males and oestrogen in females) 1evels. Serum anti-Mullerian hormone (AMH) and inhibin-B (INHBB) were assessed in male and female patients respectively to investigate Sertoli cell function and ovarian reserve (27–30). LH, FSH, testosterone, oestradiol and AMH in patients serum were measured by electrochemiluminescence (ECLIA) using an automated analyzer (Roche COBAS®). Inhibin-B serum concentration was assessed by automated enzyme-linked immunosorbent assay (DYNEX DSX™ Automated ELISA).

Pubertal development was assessed according to Tanner staging (A, axillary hair; PH, pubic hair; G, male external genitalia; B, female breast) (31).

3 Results

3.1 Case description

Patient 1 (male) presented with hyperopia and astigmatism at age 6. Shortly after, he developed bilateral neurosensorial hearing loss requiring use of hearing aids. He was diagnosed with non-autoimmune type 1 diabetes (naT1D) at age 6.7 years old and started treatment with multiple daily insulin injections (MDI). WFS1-SD diagnosis was confirmed by genetic analysis showing a double heterozygous WFS1 gene variant (c.409_424dup16; p.Val142fs∗251 and c.1628T>G; p.Leu543Arg). At the age of 8.3 years, diabetes insipidus was diagnosed and treatment with desmopressin was started. Magnetic resonance imaging (MRI) showed OA and mild brainstem hypoplasia.

Patient 2 (female) had an unremarkable medical history except for transient mild speech delay. T1D was diagnosed at age 5.2 years. At age 8.7 years, genetic analysis identified two previously unreported likely pathogenic variants (c.316-1G>A; c.757A>T and p.Lys253Ter; splice site disruption and premature stop codon respectively). MRI showed slight atrophy of brainstem and optic nerves.

Patient 3 (male) had an unremarkable past medical history except for a transient mild motor delay. T1D was diagnosed at 9 years. Genetic confirmation of WFS1-SD was established by NGS (Next Generation Sequencing) showing a double heterozygous missense variant (c.605A>G; p.Glu202Gly and c.1289C>T; p.Ser430Leu). Optical coherence tomography (OCT) scans highlighted OA at 10.7 years of age, later confirmed by MRI.

Patient 4 (male) had a prior unremarkable medical history and was diagnosed with T1D at age 12.3 yrs. Aged 13.4, OA was confirmed at OCT scan. Gene sequencing revealed heterozygous WFS1 variants resulting in a premature stop codon and missense mutation (c.387G>A; p.Trp129 and c.1675G>C; p.Ala559Pr respectively). Reduce volume of brainstem at MRI was found.

Patient 5 (female, sister of patient 3) underwent NGS analysis at age of 10.8 years after her brother's diagnosis and the same WFS1 gene variants were confirmed. Interestingly, sequencing also reported missense heterozygous variant (c.778G>C; p.Glu260Gln) of paternal inheritance in KCNQ4 gene, associated with neurosensorial hearing loss. Her past medical history included hyperopia and strabismus requiring corrective lenses, diagnosis of bilateral congenital hypoacusia at 7.3 years of age requiring hearing aids and mild speech delay for which she underwent speech therapy. Bilateral optic nerve, pons and cerebellar atrophy were found on MRI assessment at age 10.8. At age of 11.7 years glucose intolerance was observed at mixed meal tolerance test (HbA1c 31 mmol/mol; 5%).

Patient 6 (female) presented with progressive neuromotor delay since birth and bilateral congenital cataract treated with surgery at 1.1 years. Shortly after naT1D was diagnosed, NGS analysis showed a *de novo* heterozygous WFS1 gene mutation (c2425G>A, p.Glu809Lys), previously described as pathogenetic in autosomal dominant non-classic WFS1-SD. At 3.1 years severe neurosensorial hearing loss was diagnosed and hearing aids were prescribed. Patient 6 was then referred to our centre, where an advanced hybrid closed loop system was started. Growth hormone deficit was also diagnosed and replacement therapy was started. Brain MRI showed a normal pituitary gland.

Patient 7 (female) had an unremarkable past medical history until 5.5 years of age when she presented with progressive visual impairment. OA was found at OCT assessment and genetic analysis for Leber optic atrophy was negative. At the age of 8.7 years classic WFS1-SD was diagnosed (two heterozygous variants, c.108delG in exon 2 and c.2206G>A in exon 8). At the same timepoint she presented fasting hyperglycaemia (300 mg/dl) and increased haemoglobin A1c (9.8%). The patient was transferred to our Centre for follow-up and treatment

Patient 8 (female) was born from consanguineous parents. T1D was diagnosed at 4.2 years in her country of origin (Egypt). Afterwards, her medical history was complicated by bilateral cataracts (5.5 years of age) for which she underwent surgical correction. Genetic analysis performed at 9 years of age showed the presence of a single likely pathogenic homozygous variant in WFS1 (c.2140G>C, p.Asn714Asp) confirming classic WFS1-SD. Screening for associated diseases showed mild sensorineural hearing loss. The girl was referred to our centre for periodic follow-up

Table 1 summarizes patient genotype and WFS1-SD related phenotype.

3.2 Gonadal assessment

Patient 1 showed an increased value of LH and FSH first at 12 years and 8 months of age, confirmed at yearly clinical follow-up (see Table 2). Clinical examination showed a regular progression of pubic hair and genital virilisation (according to Tanner staging). However, testicular volumes remained lower than expected for age and pubertal staging (4 cc bilaterally at last evaluation; 15 yo) and inhibin-B still remained unmeasurable. Hormonal exams were diagnostic for hypergonadotropic hypogonadism, and testosterone levels are being assessed on regular follow-up to evaluate the need for testosterone replacement therapy (see Table 2).

Patient 2 presented a pubertal delay, with first evidence of breast budding around 13 years of age. Periodical laboratory assessment showed prepubertal LH, FSH and oestradiol values compatible with Tanner staging until the last follow-up (13 years and 8 months), when pubertal progression was clinically evident (Tanner stage: A2, B2, PH3) with concomitant gonadotropin rising. However, at the same age, serum AMH values were still in the lower range, possibly suggesting a early-stage primary ovarian insufficiency (POI; see Table 2).

Patient 3 presented a normal progression of pubertal development, but a testicular asymmetry, with a right testis of 5 cc and left testis of 12 cc at 13 years and 8 months of age. The ultrasound of the right testis, performed at the same timepoint, showed initial signs of fibrosis of the seminiferous tubules. Gonadotropins, testosterone, inhibin-B and AMH values remained normal for age and pubertal stage duwholeg the all follow-up (12 y 4 m – 14 y 6 m, see Table 2).

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TABLE 1 General pathognomonic characteristics of WFS and genetic mutations of all patients.

	Sex	Origin	DM Diagnosis (age)	WFS Diagnosis (age)	Genetic	Clinical characteristics	Start liraglutide (age)
Patient 1	M	Italian	6 y 8 m	8 y	Double heterozygosity: c.409_424dup16; p.Val142fsX251 and c.1628T>G; p.Leu543Arg	DM OA DI HD	11 y 3 m
Patient 2	F	Italian	5 y 2 m	8 y 7 m	Double heterozygosity: c.316-1G>A; c.757A>T. p.L	DM OA	10 y 7 m
Patient 3	M	Italian	9 y	10 y 7 m	Double heterozygosity: c.605A>G p.G1u 202G1y e - c.1289C>T p.Ser430leu.	DM OA	12 y 3 m
Patient 4	M	Italian	12 y 3 m	13 y 4 m	Double heterozygosity: c387G>A p.Trp129X e - c.1675G>C p.Ala559Pr.	DM OA	14 y
Patient 5	F	Italian		10 y 8 m	Double heterozygosity: c.605A>G p.G1u 202G1y e - c.1289C>T p.Ser430leu.	GI OA HD	11 y 3 ms
Patient 6	F	Albanian	1 y 6 m	1 y 6 m	Dominant: c2425 G>A, p.Glu809Lys	DM CC HD GHD	3 y 3 m
Patient 7	F	Hispanic	8 y 7 m	8 y 7 m	Double heterozygosity c.108delG in exon 2 and c.2206G>A	DM OA	8 y 7 m
Patient 8	F	Egyptian	4 y	9 y	Single homozygous variant c.2140G>C, p.Asn714Asp	DM CC HD	9 y 9 m

DM, diabetes mellitus; OA, optic atrophy; DI, diabetes insipidus; HD, hearing defects; UD, renal or urological problems; GI, glucose intolerance; GHD, growth hormone deficit; CC, congenital cataract.

Patient 4 showed an increased value of LH and FSH first at 14 years of age, confirmed at yearly clinical follow-up (see Table 2). Clinical examination showed a regular progression of pubic hair and genital virilisation (according to Tanner staging). However, testicular volumes remained lower than expected for age (4 cc) and pubertal staging and inhibin-B still remained unmeasurable at last evaluation (16 year old). Hormonal exams were diagnostic for hypergonadotropic hypogonadism, but testosterone levels always remained in the normal range for age and pubertal stage, with no currently need for hormonal replacement therapy (see Table 2)

Patient 5 showed normal progression of pubertal development with appropriate gonadotropin and AMH values for age and sex at all timepoints (10 y 10 m, 11 y 8 m and 12 y 1 m; see Table 2).

Patient 6 showed an increased value of LH and FSH since the first evaluation at 3 years and 3 months of age (see Table 2). Hormonal exams, performed at the same age and repeated 6 month later, were diagnostic for early hypergonadotropic hypogonadism and AMH values confirmed the diagnosis of primary ovarian insufficiency at a very early age (see Table 2).

Patient 7, assessed at 8 year and 8 months of age, was prepubertal. Serum gonadotropins and AMH values were normal for age and sex. (see Table 2).

Patient 8 showed an increased value of LH and FSH since the first evaluation at almost 10 years of age (see Table 2). Hormonal exams, performed at 9 years and 11 months of age, were diagnostic for early hypergonadotropic hypogonadism and AMH values confirmed the diagnosis of premature ovarian insufficiency (see Table 2).

Figures 1–5 show trends of AMH in males and females, Inhibin B, and gonadotropins respectively.

4 Discussion

4.1 Gonadal dysfunction: an underrated clinical feature

Clinical presentation in WFS1-SD is often complicated by neurological, urological, psychiatric and other endocrine dysfunctions. Some of the features are under-recognized despite their significant repercussions on prognosis. In particular, among endocrinological problems, gonadal dysfunction has been described in affected patients and referred to as a suggestive clinical feature and is therefore not included in major nor minor diagnostic criteria. Cases of hypogonadotropic hypogonadism due to hypopituitarism and hypothalamic dysfunction have been classically reported since the first patient described by Wolfram in 1938 (14, 15). Nonetheless descriptions of both primary and secondary hypogonadism are increasingly found in literature (14–18). The prevalence of hypogonadism in WFS1-SD patients varies widely from different reports. Salzano et al. analysed clinical findings in 14 patients with WFS1-SD and reported a prevalence of 7.1% for both hypogonadotropic hypogonadism and hypergonadotropic hypogonadism (16). A Spanish study on a cohort of 50 individuals observed hypogonadism in 27.8% of them

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TABLE 2 Gonadal and endocrinological assessment of all patients.

	Sex	Age	LH (mU/ml)	FHS (mU/ml)	E	T (ng/ml)	AMH (mcg/L)	Inhibin B (pg/ml)	Tanner stage	Hight (SDS)	Weight (SDS)	BMI (SDS)
Patient 1	M	12 y 8 m 13 y 6 m 14 y 3 m 15 y	65,6 29,2 18,9 23,3	16,6 55,7 58 54,1		2 3,11 3,27 2,18	3,96 7,21 3,66 2,9	<7 <7 <7 <7	A2; P2-3; G2 A3; P3; G3 A3; P4; G4 A3; P5; G4	1.01 0.56 0.13 0.25	0.83 0.13 -0.52 -0.69	0.48 -0.30 -0.88 -0.78
Patient 2	F	10 y 8 m 11 y 11 y 4 m 12 y 4 m 13 y 8 m	<0.3 <0.3 <0.3 <0.3 1.1	2.7 1.8 3.1 2.8 5.1	< 5 14		0.83 0.57 0.22		A1; B1; P1 A1; B1; P1 A1; B1; P1 A1; B1; P1-2 A2; B2; P3	-0.09 -0.06 -0.31 -0.54 0.10	-1.39 -1.76 -1.77 -2.16 -1.95	-2.07 -2.76 -2.55 -2.87 -2.69
Patient 3	M	12 y 4 m 13 y 1 m 13 y 8 m 14 y 6 m	1,5 4,5 6.3 4.7	1.8 2.1 3 3.3		0,38 1,69 2,93 3,63	22.5 7.77	161,9	A2; P2; G2 A2; P2; G2 A3; P3; G3 A3; P3; G3	-0.92 0.90 -0.99 -1.13	0.87 0.77 1.15 1.03	1.59 1.47 1.92 1.83
Patient 4	M	14 y 14 y 7 m 15 y 3 m 16	13.1 12.9 10.3 19.8	33.7 32.9 29.2 33.6		5.54 4.77 3.78 6.51	12.7 11.1 8.29	<7 <7 <7	A2; P2; G3 A3; P3; G4 A3; P4; G5 A3; P4; G5	1.02 0.71 0.57 0.56	0.05 -0.50 -0.72 -0.45	-0.74 -1.12 -1.37 -0.81
Patient 5	F	10 y 10 m 11 y 8 m 12 y 1 m	9.1 7 11.3	6.2 10.1 9	43 56		1.55 1.25		A2; B2-3; P2 A2; B3; P3 A3; B3; P3-4	0.94 0.73 0.69	0.04 -0.34 -0.80	-0.53 -0.97 -1.60
Patient 6	F	3 y 3 m 3 y 9 m	5.2 3.3	44.5 35.3	<5 <5		<0,01 <0,01		A1; B1; P1 A1; B1; P1	-3.10 -3.10	-3.23 -4.30	-1.82 -2.06
Patient 7	F	8 y 8 m	<0.3	1.9	<5		3.62		A1; B1; P2	-0.78	-1.61	-1.79
Patient 8	F	9 y 11 m	24.6	37.4			0.01		A1; B1; P1	-0.73	-0.95	-0.83

LH, Luteinizing hormone; FSH, Follicle-Stimulating hormone; AMH, Anti- Mullerian hormone; BMI, Body Mass Index; SDS, Standard Deviation Score.

(19). Simsek et al. described gonadal failure in about a third of WFS1-SD cases (20). Importantly, the low prevalence of the disease and heterogeneous clinical presentation makes it difficult to establish an accurate frequency of associated manifestations. Gonadal dysfunction may manifest subtly and variably as well. To date, gonadal failure has been observed more frequently in young male adults with heterogeneous clinical features (atrophic testes, small penis, erectile dysfunction, gynecomastia and poor secondary sexual characteristics) (2, 5, 15, 16, 19, 21). Females may often present primary amenorrhoea with low gonadotropins (secondary hypogonadism) and a review from 2018 describes normal ovarian function in female WFS1-SD patients (22). The reason for differences in gender in term of gonadal function is yet to be elucidated. Interestingly the first case of primary hypergonadotropic hypogonadism in a female patient with confirmed WFS1-SD diagnosis has been recently described in a 16 year adolescent (23). Moreover, pubertal delay is often observed in WFS1-SD adolescents alongside with growth delay due to concomitant hypopituitarism (15, 23). Nevertheless examples of successful pregnancy and delivery have been described and first reports of male fertility date back to 2013 (15, 24, 25).

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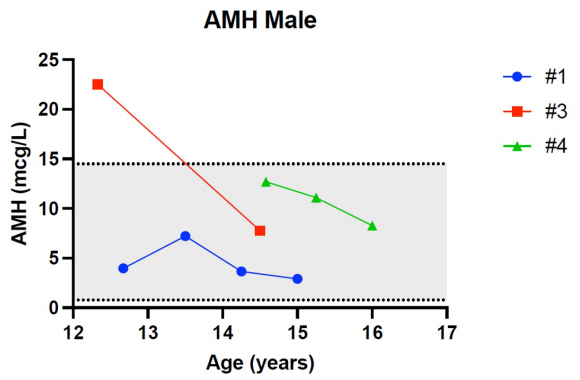

FIGURE 1
Trend of anti-Mullerian hormone in males. # stands for "patient".

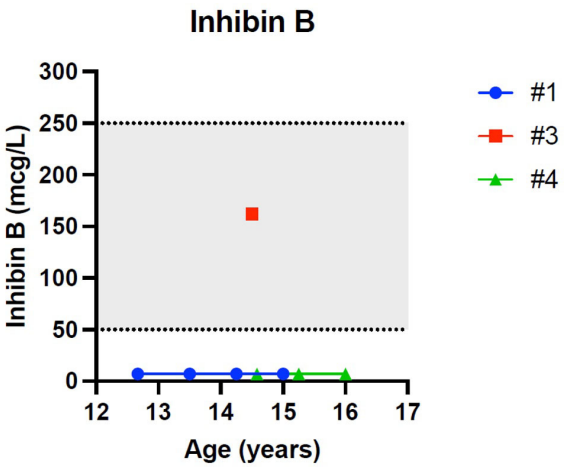

FIGURE 3
Trend of inhibin-B in males. # stands for "patient".

4.2 Pathogenesis of gonadal dysfunction in WFS1-SD

Hypogonadism in Wolfram patients has been classically attributed to hypothalamic dysfunction (secondary or hypogonadotropic hypogonadism). However, recent reports have suggested that primary hypogonadism (hypergonadotropic hypogonadism) may also be part of the spectrum (14, 15, 32). Currently, the pathogenetic mechanism underlying primary gonadal failure observed in some patients remains unclear and cannot possibly be attributed to neurodegeneration or uncontrolled diabetes. However, a first hypothesis about wolframin's role in gonadal failure has been suggested. Noormets et al. investigated the role of WFS1 gene in infertility using a murine model (33). They generated WFS1-deficient (WFS1KO) male mice and showed that reduced fertility may be related to changes in sperm morphology and reduced number of spermatogonia and Sertoli cells. On the contrary, Leydig cell number and morphology, serum testosterone and FSH concentrations did not differ between WFS1KO mice and wild-type. It is yet unclear whether wolframin underexpression impairs spermatogenesis by consequently increasing ER-stress and spermatogenic cell apoptosis. Further research is needed to clarify the mechanism through which WFS1 mutations affect gonadal cells function. Das et al. enrolled and studied clinical progression in five patients (three males, two females) with genetically confirmed WFS1-SD at a single tertiary care centre in India (17). They observed pubertal delay in 60% of patients, with primary amenorrhoea (with low gonadotropins) in both female patients (one requiring hormone therapy for pubertal initiation) and primary gonadal failure in two of the three male patients. Both of the latter showed elevated levels of gonadotropins at hormonal analysis and hormonal replacement therapy was started. Testicular biopsy revealed partly hyalinised seminiferous tubules and prominence of Leydig cells. Immunohistochemical analysis confirmed the presence of mutated wolframin, which was not significantly different from normal testis specimens on protein quantification. This may suggest gonadal cell toxicity possibly mediated by a dysfunctional protein and consequent ER-stress, as extensively described for β-pancreatic and neural cells. Although data is currently scarce, the recently proposed association between severe phenotypes and mutations causing intracellular retention, rather than loss of protein, may be suggested by reported gonadal findings (21).

These data highlight significant differences between WFS1-SD human patients and evidence from mice models with regard to laboratory and histological findings.

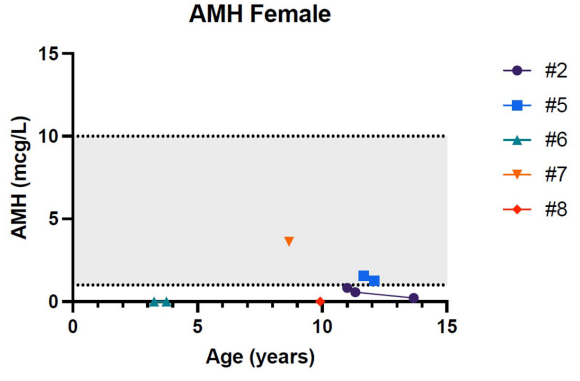

FIGURE 2
Trend of anti-Mullerian hormone in females. # stands for "patient".

4.3 Our cohort

To our knowledge this is the first case series investigating gonadal function in paediatric patients suffering from WFS1-SD.

Despite the small cohort, consistent with the rareness of WFS1-SD, primary hypogonadism was diagnosed in 50% of patients (n=4), more specifically 67% (n=2) of males and 40% of females (n=2). These data confirm that gonadal dysfunction may be a frequent and underdiagnosed clinical feature in WFS1-SD. In particular, hypergonadotropic hypogonadism seems to be more common than previously described, involving both male and female patients. Underdiagnosis may have previously been due to short life

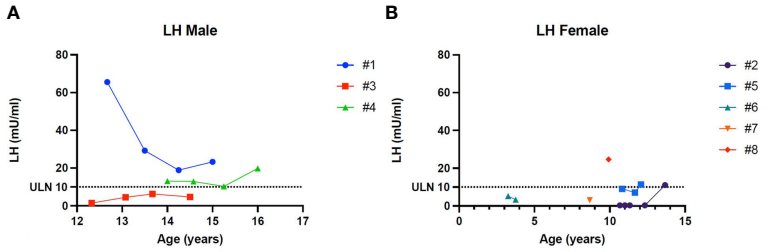

FIGURE 4
(A) Trend of the Luteinizing hormone in males. **(B)** Trend of the Luteinizing hormone in females. # stands for "patient".

expectancy and other more life-threatening, life-worsening, and therefore alluring clinical features.

4.3.1 Gonadal dysfunction seems more frequent in males than in females

Patient 1 and Patient 4 were diagnosed with hypergonadotropic hypogonadism with a concomitant undetectable level of inhibin B, highlighting the early dysfunction of Sertoli cells, which may therefore be more affected than testosterone producing Leydig cells. In fact, patient 1 showed normal testosterone until the age of 14 years and Patient 4 had normal testosterone at the latest follow-up.

Patient 3 had a normal hormonal assessment (LH, FSH and inhibin B values) and pubertal progression. Interestingly patient 3 is the male sibling of the female Patient 5, who also presented a normal hormonal assessment including AMH and pubertal progression, suggesting a normal ovarian reserve for age and sex. This may suggest that there is a specific genotype-phenotype correlation with gonadal dysfunction but further studies on larger populations are needed to confirm this hypothesis.

Our data suggest that primary hypogonadism occurs more frequently and at a younger age in female subjects than their male counterparts. This may suggest that ovarian tissue may be more affected by wolframin dysfunction than testicular tissue.

Only one case of female primary gonadal failure in patients affected by WS has been described in literature so far, as reported by Jodoin et al. in a young adolescent patient (23). To determine if a correlation between the genotype and the type and onset of clinical manifestations in WS exists, also including sexual development issues, is complicated by the rarity of the disease.

Our experience suggests that hypergonadotropic hypogonadism in WS1 is more frequent than previously reported. Furthermore, this clinical feature seems to be even more common than hypogonadotropic hypogonadism, contrary to what has been previously described.

5 Conclusions

Our case series suggests that gonadal dysfunction, with potentially significant morbidity and impaired quality of life, represents a frequent and early clinical feature in WFS1-SD. The mechanisms underlying wolframin defects and gonadal failure and possible phenotype-genotype correlations have yet to be clarified. Since gonadal function appears to be a significant, not just "suggestive" feature of the disease, our proposal is that gonadal dysfunction should be included amongst clinical diagnostic criteria, as has already been proposed for urinary dysfunction (18, 34). Early diagnosis of hypogonadism may assist in an earlier clinical suspect, allowing timely diagnosis and follow-up and care of treatable associated diseases in these young patients, including hormonal replacement therapy when needed (35, 36). Periodical assessment of gonadal function and pubertal development in every patient affected by WFS1-SD is therefore also fundamental, starting from very early age. Considering that WFS-1 mutations have been described in patients with subtle clinical features and that signs and symptoms of WFS1-SD are extremely variable, the existence of patients with gonadal dysfunction as the only presenting sign cannot be ruled out. Currently, the presence of two pathological

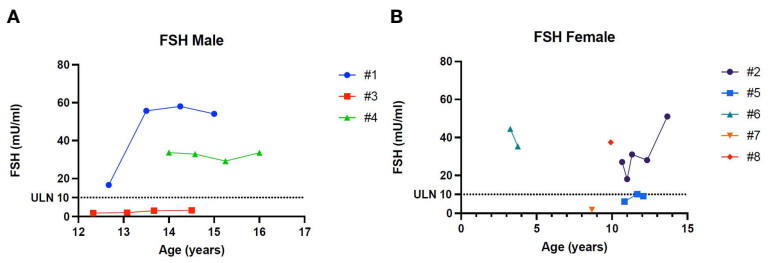

FIGURE 5
(A) Trend of Follicle-stimulating hormone in males. **(B)** Trend of Follicle-stimulating hormone in females. # stands for "patient".

mutations in WFS1 is considered a sufficient criteria for diagnosis (according to EUROWABB guidelines) (37). Therefore, the presence of gonadal dysfunction alongside other WFS1-SD clinical features should be a significant lead towards genetic testing.

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

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

GF, RT, and MS conceived the idea and wrote the manuscript. FA wrote the manuscript. All authors supervised the findings of this work. 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.

Publisher's note

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Accuracy and impact on quality of life of real-time continuous glucose monitoring in children with hyperinsulinaemic hypoglycaemia

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Objective: Continuous glucose monitoring (CGM) is the standard of care for glucose monitoring in children with diabetes, however there are limited data reporting their use in hyperinsulinaemic hypoglycaemia (HH). Here, we evaluate CGM accuracy and its impact on quality of life in children with HH.

Methods: Real-time CGM (Dexcom G5 and G6) was used in children with HH aged 0-16years. Data from self-monitoring capillary blood glucose (CBG) and CGM were collected over a period of up to 28days and analysed. Quality of life was assessed by the PedsQL4.0 general module and PedsQL2.0 family impact module, completed by children and their parents/carers before and after CGM insertion. Analysis of accuracy metrics included mean absolute relative difference (MARD) and proportion of CGM values within 15, 20, and 30% or 15, 20, and 30 mg/dL of reference glucose values >100 mg/dL or ≤100 mg/dL, respectively (% 15/15, % 20/20, % 30/30). Clinical reliability was assessed with Clarke error grid (CEG) analyses.

Results: Prospective longitudinal study with data analysed from 40 children. The overall MARD between reference glucose and paired CGM values (n=4,928) was 13.0% (Dexcom G5 12.8%, Dexcom G6 13.1%). The proportion of readings meeting %15/15 and %20/20 were 77.3% and 86.4%, respectively, with CEG analysis demonstrating 97.4% of all values in zones A and B. Within the hypoglycaemia range (<70 mg/dL), the median ARD was 11.4% with a sensitivity and specificity of 64.2% and 91.3%, respectively. Overall PedsQL child report at baseline and endpoint were 57.6 (50.5 – 75.8) and 87.0 (82.9 – 91.2), and for parents were 60.3 (44.8 – 66.0) and 85.3 (83.7 – 91.3), respectively (both p<0.001).

Conclusion: Use of CGM for children with HH is feasible, with clinically acceptable accuracy, particularly in the hypoglycaemic range. Quality of life

measures demonstrate significant improvement after CGM use. These data are important to explore use of CGM in disease indications, including neonatal and paediatric diabetes, cystic fibrosis and glycogen storage disorders.

KEYWORDS

hyperinsulinism, continuous glucose monitoring, hypoglycaemia, neurodevelopment, time below range

Highlights

- There has been limited research into the accuracy of real-time continuous glucose monitoring (CGM) in children at high risk of hypoglycaemia due to hyperinsulinaemia.
- This is the largest dataset reporting the accuracy of real-time continuous glucose monitoring in this cohort, with results demonstrating clinically acceptable accuracy, including in the hypoglycaemia range.
- Furthermore, use of CGM is associated with significant improvement in quality of life in children and their parents.
- These data are important for the use of CGM in children with rare disease indications, such as those with hyperinsulinism or rare metabolic disorders.

Introduction

Hyperinsulinaemic hypoglycaemia (HH) is caused by dysregulation of insulin secretion from pancreatic β-cells (1). It is the main cause of persistent hypoglycaemia in neonates and infants, putting them at significant risk of permanent brain damage and even sudden death (2). Children with HH are at high risk of neurological deficit with hypoglycaemic episodes (3). The long-term effects of neonatal and childhood hypoglycaemia include an impact on visual-motor integration, motor skills, and academic attainment (4). Neonatal hypoglycaemia is associated with a two-to-three-fold increased risk of specific cognitive deficits in early childhood (2-5years), and general cognitive impairment and literacy and numeracy problems in later childhood (6-11years) (5). In 60 children with hyperinsulinism followed for a period of 5 years, 46.7% of them had at least one form of neurodevelopmental delay (6).

The care and management of children with HH can be complex (7). Regular capillary blood glucose measurement (CBG) by heel or finger prick is the standard of care to monitor blood glucose concentration in the hospital as well as at home, supporting the identification and management of hypoglycaemic episodes. However, blood glucose levels may need to be measured often, and up to every 10-15 minutes at the time of hypoglycaemia, especially where children are unable to communicate the symptoms of hypoglycaemia.

Continuous glucose monitoring (CGM) provides information on real-time glucose concentration, the rate and direction of change, and alerts and alarms for predicted, or established, hypoglycaemia below customisable thresholds (8). CGM is used in children with type 1 diabetes and is supported by a robust evidence base in paediatric and adult type 1 diabetes care (9, 10). CGM may be a useful monitoring tool to identify, or even predict, hypoglycaemia in HH, with potential to reduce the burden of CBG monitoring and the discomfort and pain associated with it. Moreover, CGM may enable identification of otherwise undetected episodes of hypoglycaemia especially those with asymptomatic hypoglycaemia and may have the potential to reduce the risk of neuroglycopenia in the developing brain.

There is limited evidence supporting CGM accuracy in the low blood glucose range in babies and young children, and little data supporting CGM use in children with HH (11). Previous studies in children with HH showed that CGM (Dexcom G4-G6) tends to under read compared with CBG measurements (12, 13). In another study, CGM (Dexcom G6) demonstrated an over reading compared to CBG (14), similar to another study with Freestyle Libre 1 (15). However, CGM can be used to help to understand glycaemic patterns and support prevention of severe hypoglycaemia (16).

In order to explore the potential for CGM to attenuate exposure to hypoglycaemia in children with HH, and thereby possibly reducing the risk of neurodevelopmental delay, data supporting the feasibility and accuracy of CGM in this population are required. The aim of this study is to determine the accuracy of real-time CGM in children with HH and to report pilot quality of life outcomes associated with CGM use.

Methods

Study design and participants

This is a prospective longitudinal study in children with HH conducted in both inpatient and home environment settings. Dexcom (San Diego, California) G5 and G6 CGM systems were used. The inclusion criteria were children with a diagnosis of HH within the age range of term babies with corrected gestational age >37weeks up to 16 years of age. Recruited neonates were over 2kg. The Dexcom G6 CGM system was used in children aged above 2 years. For children aged between 0-2 years, the Dexcom G5 system was used, as the G5 sensor has a manual inserter which enables the

angle and force of the sensor insertion to be adjusted for smaller participants.

Parents and carers were requested to do at least six CBG readings by heel or finger prick sampling every 24 hours (fasting, pre-meals and before bedtime), and were supported to continue usual hypoglycaemia management during the duration of the study, based on CBG results. CBG was assessed using an ISO 15197:2013 compliant CBG device in line with the manufacturers' instructions (Accu-Chek Inform II in an inpatient setting or with Accu-Chek Performa Nano glucometers at home). If the the CGM glucose was reported to be <72mmol/L (<4 mmol/L), the participant, carer or parent checked CBG concentration. If the initial capillary blood glucose was <63mg/dL (3.5mmol/L), a repeat sample was taken immediately from another site as per standard of care in children with HH. If the second value was <63mg/dL (3.5mmol/L), then this was treated as hypoglycaemia. All capillary blood glucose values along with CGM value were documented in a diary.

Parents and carers were taught how to insert and remove the sensor, and written information with emergency contact details were given to parents. Sensors for Dexcom G5 were changed every 7 days and Dexcom G6 every 10 days. Parents of children using the Dexcom G5 (<2 years old) were required to calibrate every 12 hours as per manufacturer guidance. For users of Dexcom G6, there was no requirement to calibrate the sensor.

Participants remained in the study for a period of 1 to 4 weeks. Glucose data were downloaded from all devices at the end of the study period. CBG readings were paired to the nearest 5 minute CGM value (either before or after). Ethical approval was granted by the London Fulham NHS Research and Ethics Committee.

Quality of life study questionnaires

Age-appropriate and validated quality of life questionnaires (PedsQL version 2.0 for parents of participants aged 2-4 years, and PedsQL version 4.0 to all participants >4 years of age) were completed by participants and their families at the start and end of the monitoring period.

The PedsQL Generic Core Scales have child self-reporting forms designed for ages 5–7 (young child), 8–12 (child), and 13–18 (adolescent) years. Children >8 years report how much of a problem each item has been for them during the past one month. PedsQL assessments consists of 4 subscales on physical, emotional, social and school functioning, and include a Likert response scale (0 = never a problem; 1 = almost never a problem; 2 = sometimes a problem; 3 = often a problem; 4 = almost always a problem) for each item in each scale. A simplified, 3-point rating scale was used for younger children (0 = not at all; 2 = sometimes; 4 = a lot). Items were reverse-scored and transformed to a 0–100 scale where higher scores indicate better health related quality of life (i.e. 0 = 100, 1 = 75, 2 = 50, 3 = 25, 4 = 0). Scale scores were computed as the sum of the items divided by the number of items answered (this accounts for missing data). If greater than 50% of the items in the scale were missing, the scale score was not computed. A detailed description of scoring can be found via the PedsQL™ website (17).

Primary and secondary outcomes

The primary endpoint was the overall mean absolute relative difference (MARD; %) between CGM compared to the reference CBG glucose. Secondary endpoints included median absolute relative difference (median ARD, %) for CGM and in the predefined glycaemic ranges: <70 mg/dL (<3.9 mmol/L); <63 mg/dL (<3.5 mmol/L); 70-180 mg/dL (3.9-10 mmol/L); >180 mg/dL (>10 mmol/L). The UK consensus in the management of hypoglycaemia in people with congenital hyperinsulinaemia is 63 mg/dL (3.5 mmol/L) (14).

Additional endpoint measures include Clarke error grid analysis, as well as percentage (%) time in range (TIR; 70–180 mg/dL; 3.9-10 mmol/L), % time in hypoglycaemia (<70 mg/dL; <3.9 mmol/L), and hyperglycaemia (>180 mg/dL; >10 mmol/L) calculated for the total study period.

Statistical methods and data analysis

Glucose CGM sensor performance was evaluated by the absolute relative difference determined as an aggregate value from the total number of paired points. The performance evaluation also included the proportion of the CGM system values that are within ±20% of relative difference of reference value at glucose levels > 100 mg/dL (>5.6 mmol/L) and ±20 mg/dL (± 1.1 mmol/L) of absolute difference at glucose level ≤ 100 mg/dL (≤ 5.6 mmol/L), hereafter referred to as % 20/20, as well the proportion of the CGM system values that are within ± 15% of relative difference of reference value at glucose levels >100 mg/dL (>5.6mmol/L) and ±15 mg/dL (± 0.8 mmol/L) of absolute difference at glucose level ≤ 100 mg/dL (≤ 5.6 mmol/L), hereafter referred to as % 15/15. Sensitivity and specificity was also calculated for each hypoglycaemic threshold of <70mg/dL (<3.9mmol/L), <63mg/dL (<3.5mmol/L) and <54mg/dL (<3.0mmol/L), as well as the positive predictive value and negative predictive value.

Clarke error grid (CEG) analyses (18) were used to quantify the clinical accuracy of the CGM sensors, with Bland–Altman plots used to depict the data distribution and bias between sensor and the reference glucose. Measures of glycaemic variability were computed using EasyGV (v10.0) software (19).

Data have been presented as mean (standard deviation) and median (interquartile range [IQR]), unless otherwise stated. Distribution of data were assessed by quantile-quantile and density plots indicating non-normal distribution. Thus, the median ARDs have been reported alongside the MARD. Statistical tests were two-tailed and results considered statistically significant if $p<0.05$. Statistical analysis was performed using Stata version 15 (StataCorp, College Station, TX).

Results

The study was conducted from June 2019 to March 2020 in a Paediatric Endocrine centre in London. There were 44 participants diagnosed with HH recruited in the study. Four participants were

withdrawn from the study; one child developed mild skin rash at the sensor site and one developing swelling at the sensor insertion site. Two other participants did not submit CBG data to compare and were therefore withdrawn. Data from 40 children ranging from 0-16 years were included in the analysis.

There were 35 children aged >2 years old using Dexcom G6 and 5 children aged <2 years old using Dexcom G5. Twenty-seven (67.5%) children had a genetic cause identified, with remaining 13 (32.5%) having an unknown cause of HH. Baseline characteristics for the participants are summarized in Table 1. The mean sensor wear time for all participants was 21.2 ± 7.6 days.

CGM accuracy

There were 4,928 CGM data points with paired CBG values (4,074 for Dexcom G6 and 854 for Dexcom G5). The MARD between paired CGM and CBG values was 13.0% (13.1% for Dexcom G6 and 12.8% for Dexcom G5; Table 2). For CBG values <3.9mmol/L (n= 779), the median ARD was 11.4% (11.7% for Dexcom G6 [n=599] and 10.5% for Dexcom G5 [n=180]). For clinically relevant hypoglycaemia (<63mg/dL; <3.5mmol/L) for participants with HH, the median ARD was 14.3% (11.6% for Dexcom G6 [n=288] and 14.3% for Dexcom G5 [n=74]). The

TABLE 1 Baseline demographics for recruited participants (n=40).

Characteristics of the participants	Dexcom G6 (n=35)	Dexcom G5 (n=5)	Combined (n=40)
Age			
0-2 years	–	5 (100.0)	5 (100.0)
2-4 years	13 (37.1)	–	13 (37.1)
5-7 years	14 (40.0)	–	14 (40.0)
8-12 years	6 (17.1)	–	6 (17.1)
13-16 years	2 (5.7)	–	2 (5.7)
Gender			
Male Female	17 (48.6) 18 (51.4)	2 (40.0) 3 (60.0)	19 (47.5) 21 (52.5)
Diagnosis			
HI (unknown)	13 (37.4)	–	13 (32.5)
HI ABCC8	12 (34.3)	1 (20.0)	13 (32.5)
HI PMM2	2 (5.7)	–	2 (5.0)
HI GLUD1	2 (5.7)	1 (20.0)	3 (7.5)
HI HNF4α	2 (5.7)	1 (20.0)	3 (7.5)
HI Beckwith Wiedeman Syndrome	1 (2.9)	1 (20.0)	2 (5.0)
HI Fanconi Syndrome	1 (2.9)	–	1 (2.5)
HI Kabuki Syndrome	1 (2.9)	–	1 (2.5)
HI Von Willebrand disease Type	1 (2.9)	–	1 (2.5)
HI ZC4H2 Wieacker-Wiff syndrome	–	1 (20.0)	1 (2.5)
Treatment			
Diazoxide	8 (22.9)	1 (20.0)	9 (22.5)
Diazoxide ± other (i.e. octreotide/nifedipine)	11 (31.4)	1 (20.0)	12 (30.0)
Lanreotide	9 (25.8)	1 (20.0)	10 (33.3)
Octreotide	6 (17.1)	1 (20.0)	7 (17.5)
Not on any medication	1 (2.9)	1 (20.0)	2 (5.0)
Feeds			
Oral Feeds	28 (80.0)	3 (60.0)	31 (77.5)
Oral feeds and Gastric feeds	7 (20.0)	2 (40.0)	9 (22.5)

Results are expressed as median (IQR) or n (%). ABCC8, ATP Binding Cassette Subfamily C Member-8; GLUD1, glutamate dehydrogenase-1; HI, hyperinsulinism; HNF4α, hepatocyte nuclear factor-4α; PMM2, phosphomannomutase-2; ZC4H2, Zinc finger C4H2-type containing gene.

overall %15/15 and %20/20 agreement was 77.3% and 86.4%, respectively (Dexcom G6: 77.1% and 86.3% respectively; Dexcom G5: 77.9% and 86.9% respectively).

On the Clarke error grid analysis, 82.3% of points fell in the clinically accurate zone A, with 97.4% of all points falling in zones A and B (Figure 1A). Clarke error grids for Dexcom G5 and G6 are available as Supplementary Material. As illustrated on the Bland Altman analyses (Figure 1B), mean bias was +1.4mg/dL (+0.08mmol/L), with 95% limits of agreement for CGM to reference glucose CBG were -33.3mg/dL (-1.85mmol/L) and +36.1 mg/dL (+2.01mmol/L).

For the combined sensor data, sensitivity and specificity for hypoglycaemia (<63 mg/dL; <3.5 mmol/L) were 49.2% and 94.9% respectively, and 40.7% and 98.2% for hypoglycaemia (<54 mg/dL; <3.0mmol/L). When the hypoglycaemia threshold was increased to 70 mg/dL (3.9 mmol/L), sensitivity and specificity were 64.2% and 91.3% respectively. For the hypoglycaemia threshold of 70mg/dL (3.9mmol/L), the positive predictive value was 55% and the negative predictive value was 90% (Table 3).

Times in glycaemic range and variability

Times in glycaemic range for children with HH are demonstrated in Table 2. The median (IQR) %TIR 70-180 mg/dL (3.9 – 10 mmol/L) for Dexcom G6 was 94.5 (86.9-96.8)% and Dexcom G5 was 83.8 (76.9-88.5)%. Percentage time below range <70 mg/dL (<3.9 mmol/L) was 2.6 (0.6-5.9)% for Dexcom G6 and 14.5 (11.5-21.4)% for Dexcom G5. For clinically relevant hypoglycaemia in HH, the percentage time below range <63mg/dL (<3.5mmol/L) for Dexcom G6 and G5 was 0.9 (0.2-2.1)% and 4.7 (3.6-13.5), respectively. Percentage time above range 180 mg/dL (>10 mmol/L) was 1.2 (0.5-5.6)% and 1.3 (0.7-1.3)% for Dexcom G6 and G5, respectively. The coefficient of variance was 23.2 (19.2-28.2)% and 27.5 (26.1-33.7)% for Dexcom G6 and G5, respectively.

Quality of life outcomes

Quality of life questionnaires were available for 22 children aged >5 years old and family impact reports for 30 participants; remaining questionnaires were incomplete. Overall child reported PedsQL scores at baseline and after CGM use were 57.6 (50.5 – 75.8) and 87.0 (82.9 – 91.2) respectively, for all age groups (p<0.001; Table 4). Subgroup analyses for child reported PedsQL scores at baseline and endpoint were 56.5 (48.9 – 69.0) and 90.0 (86.5 – 91.3) for 5 to 7 years old (n=14; p=0.001); 75.5 (59.7 – 81.8) and 83.2 (76.1 – 83.7) for 8 to 12 years old (n=6; p=0.046); 63.0 (56.5 – 69.7) and 88.0 (87.0 – 89.1) for 13 to 16 years old (n=2).

Overall parent reported scores at baseline and after CGM use were 60.3 (44.8 – 66.0) and 85.3 (83.7 – 91.3) respectively, for

TABLE 2 Sensitivity and specificity for varying glycaemic threshold using Dexcom G5 (n=854), G6 (n=4,074) and the combined dataset (n=4,928).

	Dexcom G6	Dexcom G5	Combined
Measures of accuracy			
n paired data points	4074	854	4928
Mean ARD (%)	13.1 (± 13.6)	12.8 (± 12.5)	13.0 (± 13.4)
Median ARD (%)	9.3 (4.8 – 17.0)	9.4 (4.0 – 17.0)	9.3 (4.8 – 17.0)
Median ARD (%) <63mg/dL (<3.5mmol/L)	14.3 (7.3 – 23.5)	11.6 (5.8 – 21.1)	14.3 (7.0– 23.3)
Median ARD (%) <70mg/dL (<3.9mmol/L)	11.7 (5.8 – 21.7)	10.5 (3.4 – 19.7)	11.4 (5.6 – 21.2)
Median ARD (%) 70 -180mg/dL (3.9-10mmol/L)	8.7 (4.4 – 15.2)	9.1 (4.0 – 16.1)	9.1 (4.5 – 16.3)
Median ARD (%) >180 mg/dL (>10 mmol/L)	6.0 (3.9 – 9.1)	8.7 (8.2 – 24.5)	6.1 (3.9 – 9.2)
%15/15 (%)	77.1	77.9	77.3
%20/20 (%)	86.3	86.9	86.4
Glycaemic outcomes			
Times in range, %			
<54mg/dL (<3.0mmol/L)	0.2 (0.0-0.6)	1.0 (0.8-4.9)	0.2 (0.0-0.8)
<63mg/dL (<3.5mmol/L)	0.9 (0.2-2.1)	4.7 (3.6-13.5)	1.0 (0.2-3.2)
<70mg/dL (<3.9mmol/L)	2.6 (0.6-5.9)	14.5 (11.5-21.4)	2.9 (1.2-7.5)
70 -180mg/dL (3.9-10mmol/L)	94.5 (86.9-96.8)	83.8 (76.9-88.5)	92.9 (85.5-96.6)
>180mg/dL (>10mmol/L)	1.2 (0.5-5.6)	1.3 (0.7-1.3)	1.3 (0.5-4.0)
>270mg/dL(>15mmol/L)	0.2 (0.0-0.6)	0.1 (0.0-0.1)	0.0 (0.0-0.2)
Average blood glucose, mmol/L	6.1 (5.8-6.7)	5.0 (4.9-5.6)	6.0 (5.6-6.7)
CV, %	23.2 (19.2-28.2)	27.5 (26.1-33.7)	24.1 (19.2-29.0)
MAG	2.4 (2.2-2.9)	2.4 (2.4-2.8)	2.4 (2.2-2.9)

Results are expressed as median (IQR)

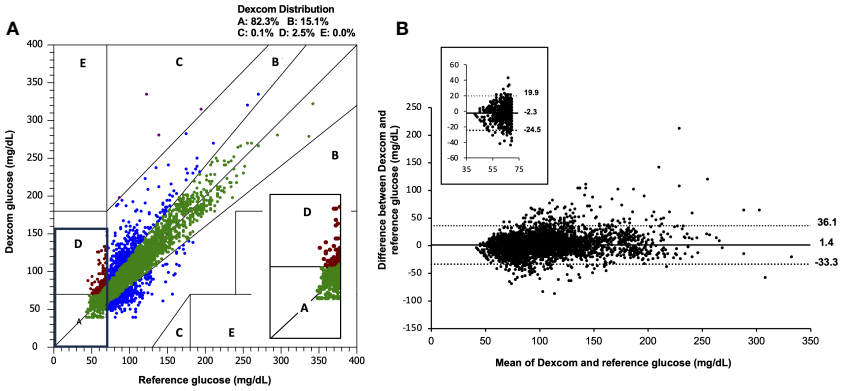

FIGURE 1
(A) Clarke error grid and **(B)** Bland Altmann analysis for combined Dexcom data (n=4,928) compared with capillary blood glucose. Inset images demonstrate the range of values observed when reference and sensor values are <70 mg/dL (3.9 mmol/L). The overall mean bias for Dexcom G6 was +1.4 mg/dL (+0.08mmol/L) and 95% limits of agreement were -33.3mg/dL (-1.85mmol/L), +36.1 mg/dL (2.01mmol/L). CBG, capillary blood glucose.

children of all age groups (p<0.001). Family impact (parent reported) scores were 45.4 (27.4 – 58.0) and 80.6 (76.8 – 84.8) at baseline and endpoint, respectively (p<0.001).

Safety outcomes

No serious device-related adverse events occurred during the study, apart from one child who developed mild skin rash at the sensor site and one child developed swelling at the sensor insertion site.

Discussion

This is the largest study demonstrating accuracy and feasibility of real-time CGM for children with hyperinsulinaemic hypoglycaemia, including in the hypoglycaemic range. Furthermore, use of real-time CGM has a beneficial impact on the quality of life for children and their parents/carers.

The combined accuracy of all paired data points demonstrated an overall MARD of 13.0% across all clinically relevant glucose ranges, including the hypoglycaemic range. The MARD is slightly higher compared to that observed in accuracy studies in children with diabetes with Dexcom G5 and G6 (10% and 9%, respectively), likely due to more sensor data in this study being in the low and low-normal glucose range. On CEG analysis, 97.4% of values lie within zones A and B, meeting the established criteria that >95% of readings lie in either zone A or B (20). There was no systematic bias in the difference between CGM and CBG levels. Our sensor data are in keeping with previously reported results for hypoglycaemia sensitivity in CHI, which reportedly range from 43-73% (12–14).

Evidence from people with diabetes suggests that CGM is effective for people at high risk of hypoglycaemic events (21, 22), including people with impaired awareness of hypoglycaemia and recent severe hypoglycaemia requiring the assistance of a third party to treat (23, 24). Time spent in the hypoglycaemic range is consistently reduced with CGM use and the incidence of severe hypoglycaemia may be reduced (23, 25). Transferring these benefits to children with HH has potential to reduce the burden of CBG self-monitoring, reduce exposure to hypoglycaemia, including reducing the incidence of seizures associated with hypoglycaemia, and may prevent the deleterious effects of neuroglycopaenia.

The accuracy of CGM seen in the study population of children with HH is acceptable, suggesting that widespread use of CGM for children with HH has potential to be a safe, effective management tool especially in those children with asymptomatic hypoglycaemia and severe disease needing frequent monitoring. CGM may be particularly beneficial for young children who are unable to articulate symptoms, alerting parents to impending hypoglycaemia

TABLE 3 Measures of accuracy and glycaemic outcomes for Dexcom G5, G6 and the combined dataset.

	Dexcom G6 (n=4,074)		Dexcom G5 (n= 854)		Combined (n=4,928)	
Hypoglycaemia threshold	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
<70mg/dL (<3.9mmol/L)	61.9	92.3	70.4	82.8	64.2	91.3
<63mg/dL (<3.5mmol/L)	47.2	95.7	56.8	91.0	49.2	94.9
<54mg/dL (<3.0mmol/L)	40.0	98.6	43.8	96.7	40.7	98.2

Accuracy was assessed by matched CGM data points with paired CBG values (Dexcom G5 n=854; Dexcom G6 n=4,074 and the combined dataset n=4,928). Glycaemic outcomes assessed for children using Dexcom G5 (n=5), G6 (n=35) and the combined dataset (n=40). Results are expressed as median (IQR)/n (%) except where indicated. ARD, absolute relative difference; CGM, continuous glucose monitoring; CV, coefficient of variance; MAG, mean amplitude glucose.

TABLE 4 Quality of life outcomes pre- and post- CGM PedsQL report from children and their families.

Pre and Post CGM insertion PedsQL	N	Baseline (Pre-CGM)	End-point (Post-CGM)	Median change from baseline to endpoint	P value
Family Impact Parent Report	**30**	**45.4 (27.4 – 58.0)**	**80.6 (76.8 – 84.8)**	**+30.6 (17.9 – 46.1)**	**<0.001**
Overall Child Report PedsQL 5-16yrs	**22**	**57.6 (50.5 – 75.8)**	**87.0 (82.9 – 91.2)**	**+32.7 (10.1 – 38.6)**	**<0.001**
Child Report PedsQL 5-7yrs	14	56.5 (48.9 – 69.0)	90.0 (86.5 – 91.3)	+37.0 (28.6 – 41.2)	0.001
Child Report PedsQL 8-12yrs	6	75.5 (59.7 – 81.8)	83.2 (76.1 – 83.7)	+8.2 (4.3 – 14.3)	0.046
Teenager Report PedsQL 13-16yrs	2	63.0 (56.5 – 69.7)	88.0 (87.0 – 89.1)	+25.1 (19.6 – 30.4)	NA
Overall Parent Report PedsQL 5-16yrs	**22**	**60.3 (44.8 – 66.0)**	**85.3 (83.7 – 91.3)**	**+27.1 (20.2 – 41.0)**	**<0.001**
Parent Report PedsQL 5-7yrs	14	55.4 (46.5 – 63.7)	83.7 (82.9 – 90.5)	+30.4 (22.3 – 40.0)	0.001
Parent Report PedsQL 8-12yrs	6	70.1 (41.6 – 79.1)	88.0 (84.2 – 92.1)	+21.7 (7.3 – 42.7)	0.028
Parent Report PedsQL 13-16yrs	2	54.9 (49.7 – 60.1)	86.4 (86.1 – 86.7)	+31.5 (26.1 – 40.0)	NA

Results are expressed as median (IQR)/n (%). p<0.05 determines significance. Statistics not performed for teenagers aged 13-16 years old as only n=2. Text in bold are overall PedsQL scores.

with predictive alerts. Furthermore, the Dexcom Share feature allows for parents to monitor glucose levels, allowing them to intervene within a timely fashion. Support and education for parents and carers of children with HH will be required to ensure optimal prevention of hypoglycaemia without burden.

In terms of glycaemia, children using Dexcom G5 had a higher proportion of hypoglycaemia than those reported using the Dexcom G6 cohort. Although this is a smaller cohort (n=5 only), these children, aged 0 to 2 years old, also had identified genetic mutations rendering them at confirmed risk of hypoglycaemia. We note children also spend a degree of time above range (>10mmol/L; >180mg/dL), in contrast to the normal range data for children without diabetes (26), which may reflect overcorrection of hypoglycaemia. This may also explain the relatively effective treatment and low frequency of hypoglycaemia (percentage time <63mg/dL; <3.5mmol/L was 1.0%).

The major strength of the study is the population. We recruited a group of children across a wide age range with the rare and complex condition of HH in a tertiary setting. Additionally, the volume of paired data points collected are the largest and acceptable for an accuracy study, and included both hospital and home environment glucose profiles, ensuring the results are applicable to in-patient and out-patient monitoring. Furthermore, a significant proportion of paired data points were in the hypoglycaemic range, demonstrating CGM accuracy in the most clinically important range.

The main limitation of the study is missing quality of life data for some participants. Despite this, our data demonstrate a significant improvement in family impact, parent-reported quality of life, and child-reported quality of life up to the age of 13years old. These findings are in keeping with the significant morbidity, psychosocial and financial burden for children and families affected with HH (27, 28). Data were only available for two participants in the 13 to 16 years old age group.

Finally, this dataset provides further evidence for access to CGM to be considered and widened for people with recurrent hypoglycaemia due to HH. Furthermore, these results could assist with other diseases, including those with neonatal and paediatric diabetes, cystic fibrosis and glycogen storage disorders, but further research in these areas is needed. The clinical effectiveness in reducing exposure to hypoglycaemia compared to usual care can now be assessed and, critically, cost effectiveness of CGM in HH can be evaluated (29). The potential to prevent hospital admissions and reduce length of stay, along with longer-term impact of reducing disability and the direct and indirect costs associated should be considered. Future studies incorporating artificial intelligence and machine learning algorithms may further reduce the burden of hypoglycaemia (30).

Conclusion

Data from Dexcom G5 and G6 CGM systems accurately reflect CBG in HH, including at the time of hypoglycaemia. Accuracy combined with significant improvements in quality of life for children and their parents suggests that the use of CGM should be considered as a standard of care for children with HH.

Data availability statement

Data cannot be shared publicly because it is data from the National Health Service. Requests to access the datasets should be directed to nick.oliver@imperial.ac.uk and pratik.shah6@nhs.net.

Ethics statement

Ethical approval was granted by the London Fulham NHS Research and Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin.

Author contributions

MS: Data curation, Formal Analysis, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. PA: Formal Analysis, Writing – original draft, Writing – review & editing. CG: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. LD: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. KM: Data curation, Investigation, Methodology, Project administration, Writing – review & editing. NO: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. PS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1265076/full#supplementary-material

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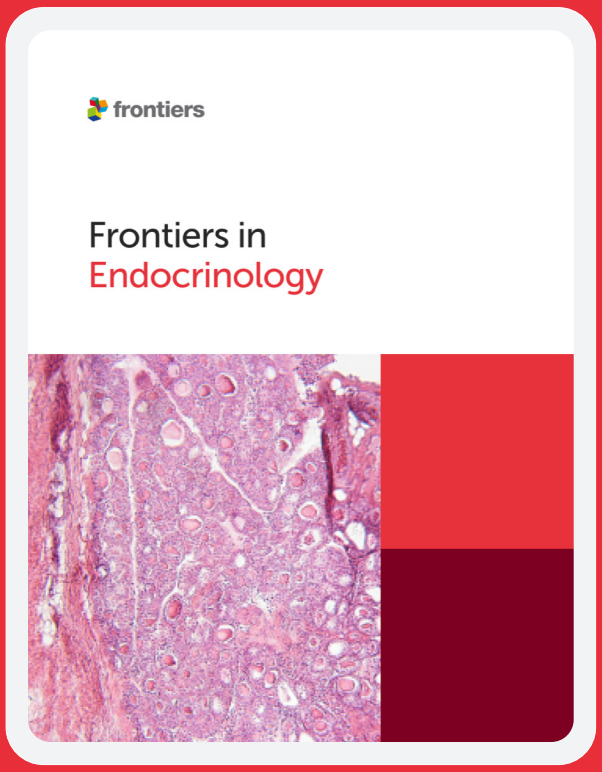

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