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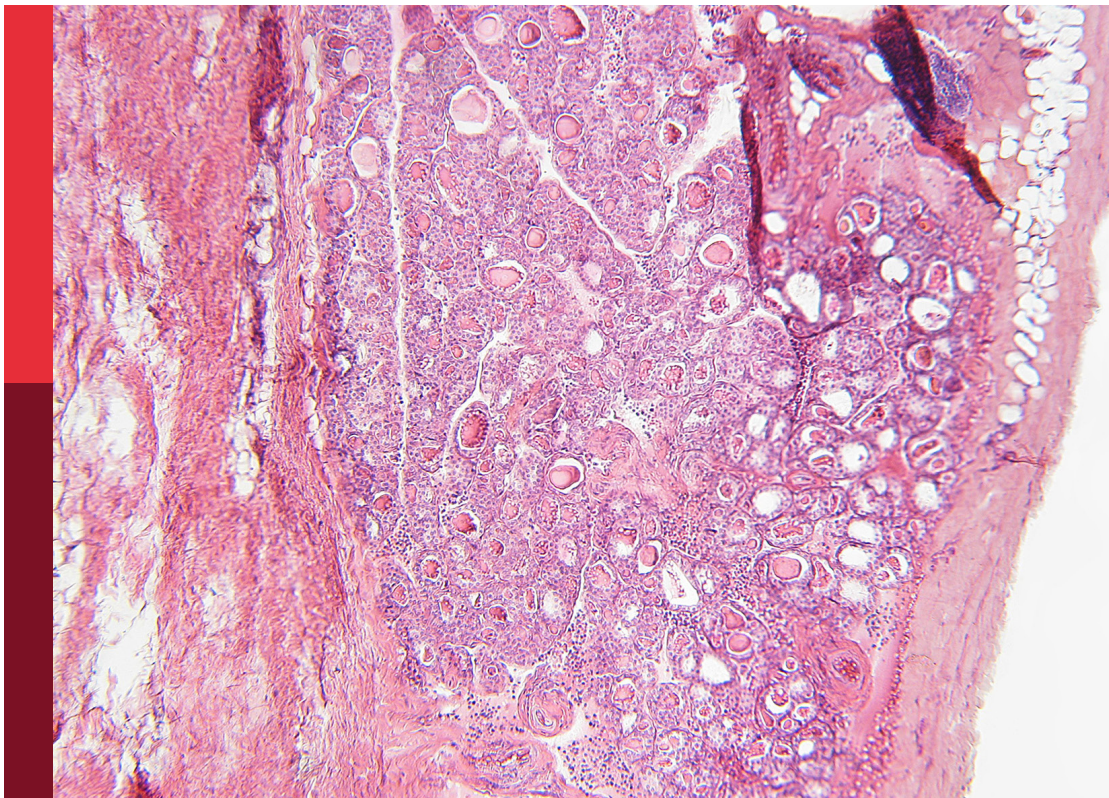

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Insights in pediatric endocrinology: 2024

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Editorial: Insights in pediatric endocrinology: 2024

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puberty, aromatase inhibitor therapy, Prader Willi syndrome, X-linked hypophosphatemia, thyroid nodules, type 1 diabetes, metabolic syndrome associated steatotic liver disease, Rett syndrome

Editorial on the Research Topic
Insights in pediatric endocrinology: 2024

This Research Topic included three papers on puberty related issues. A review by Bangalore Krishna and Garibaldi discusses assays currently used for assessment of pubertal hormones and the limitations of these assays. The review emphasizes the importance of using highly sensitive assays when relying on a single early morning basal luteinizing hormone (LH) level to diagnose central precocious puberty (CPP), and the importance of confirming this with a repeat, early morning sample. The authors point out that while pubertal levels of basal LH are diagnostic of CPP, undetectable LH levels do not exclude onset of central puberty, and that such results may occur in 20 - 35% of girls and about 5% of boys in early puberty. They point out that high dose biotin supplements can interfere with results of gonadal steroid assays, and should be discontinued if results do not make clinical sense. Finally, they point out that girls with CPP may not always demonstrate a stimulated LH level following leuprolide administration of > 5 IU/L; however, the majority will achieve peak estradiol levels of > 50 pg/mL about 20 – 24 hours after leuprolide, consistent with pubertal activation. In contrast, boys with CPP do demonstrate peak LH levels of > 5 IU/L and 20 – 24 hour testosterone levels are less important.

Further, Li et al., report on the possible contribution of perfluorinated endocrine disrupters to the increased risk of CPP in girls noted during the COVID - 19 pandemic. Using metabolomics and enrichment analysis, they demonstrate an increase in levels of three perfluorinated compounds in girls with CPP compared to prepubertal controls and involvement of multiple pathways in the CPP process. They conclude that perfluorinated compounds may promote CPP in girls by interfering with pathways that impact the hypothalamic-pituitary-gonadal axis, and urge additional research on environmental endocrine disrupters.

Finally, Ebo et al. report on the validity and reliability of self-staging of puberty, particularly in the context of the use of this strategy during televisits, using data gathered during the COVID - 19 pandemic. A kappa value of ≥ 0.60 was used to indicate significant agreement of ratings of breast and pubic hair staging in girls, and testicular size and pubic hair staging in boys as assessed by the patient and the physician. The highest kappa values were evident in girls at the extremes of pubertal staging (stages 1 and 5), while for boys, the

highest values were noted for Tanner stages 1 and 2 of puberty. Self-staging appears to work well in distinguishing between presence or absence of puberty, while being less useful in characterizing specific pubertal stages. In addition to televisits, these findings have important implications for the use of self-staging in research studies.

Zhu et al. examine associations of the inflammatory marker, high sensitivity C-reactive protein (hs-CRP), with sex steroid levels in prepubertal and pubertal children, and report inverse associations of hs-CRP with testosterone in pubertal boys and with estradiol in pubertal girls. In contrast, hs-CRP levels were positively associated with estradiol in prepubertal and pubertal boys, and with testosterone in prepubertal girls. BMI was associated positively and sex hormone binding globulin (SHBG) negatively with hs-CRP levels, consistent with higher BMI being associated with higher insulin and lower SHBG levels and greater metabolic risk. The study demonstrates that many factors regulate inflammatory and metabolic risk in youth.

Marin et al. discuss the use of MRI in pediatric endocrinology for conditions such as growth hormone deficiency, short stature and CPP, and report on the large proportion of normal scans (67%), the high prevalence of incidentalomas (17%), and that 86% of repeat scans were unnecessary based on established protocols. They recommend developing guidelines for scanning to optimize yield, reduce costs and minimize distress to patients and caregivers.

Giannopoulou et al. report on the impact of aromatase inhibitor therapy (letrozole) in a family with aromatase excess syndrome and demonstrate prevention of gynecomastia and improved adult height when letrozole therapy is initiated early, and improved physical strength and libido when this is started in adult life. Thus, aromatase inhibitors may be considered a therapeutic strategy in patients with aromatase excess syndrome.

The Research Topic includes two papers on Prader Willi syndrome. The paper by Wędrychowicz et al. explores the prevalence of central adrenal insufficiency (CAI) in children with Prader Willi syndrome using the low dose ACTH stimulation test, the glucagon stimulation test or both. Overall, only 1 of 46 children demonstrated convincing evidence of CAI, suggesting a low prevalence. The authors recommend against routine screening for CAI, and that the diagnosis should be confirmed with two tests to avoid unnecessary treatment with hydrocortisone. The second paper is a review of the condition by Madeo et al. and describes endocrine conditions in relation to specific genetic etiology of Prader Willi syndrome.

Wang et al. discuss data from a meta-analysis and systemic review of burosomab treatment in children with X-linked hypophosphatemia. The study includes data from eight cohort studies and two randomized controlled trials and concludes that burosumab has excellent therapeutic efficacy in treating this condition.

Januś et al. present a retrospective analysis of the histopathology underlying the ultrasound findings in benign, borderline, and malignant thyroid nodules in 47 children at a single tertiary thyroid center in Poland. Each type of thyroid nodule is characterized in detailed pathological terms, along with sonographic findings. The reader immediately notes the descriptive overlap in ultrasound findings among the different tumor types, leading the authors to conclude that ultrasonography is insufficient for accurate risk stratification, thereby necessitating fine-needle aspiration biopsy (FNAB) in children.

An increase in the prevalence of autoimmune diseases during the COVID - 19 pandemic has been reported in several studies, while others have not found an association. Hampering these studies was the lack of pre-pandemic control data. Hence, Herczeg et al., in a retrospective analysis, determined the prevalence of thyroid autoimmunity (TA) for the 10 years prior to the pandemic and during the pandemic in a cohort of 1,361 children and young adults with type 1 diabetes (T1D). The increase in the prevalence of anti-thyroid autoantibodies in children with T1D was detected during the pre-pandemic years but not during the COVID - 19 pandemic. Interestingly, 28.5% of children with anti-thyroid autoantibodies had clinically relevant thyroid-stimulating hormone (TSH) abnormalities (most commonly subclinical hypothyroidism) and/or were prescribed thyroid medication. They conclude that although there was a rise in the prevalence of thyroid autoimmunity among T1D children over the past decade, there was no association with the increase in the development of the disease with COVID - 19.

Type 1 diabetes is characterized by the destruction of pancreatic beta cells, which leads to insulin deficiency and significantly reduced levels of the exocrine pancreatic enzymes amylase, lipase, and trypsin. Bruggeman et al. aimed to determine whether the recently approved immunotherapies—anti-thymocyte globulin (ATG) and pegylated granulocyte colony-stimulating factor (GCSF)—resulted in changes in these three exocrine pancreatic enzymes that could serve as biomarkers to delineate response to treatment. Although the number of patients was small, there were interesting findings noted among responders to therapy (n=4-6), placebo "responders" (n=2), treated non-responders (n=16), and placebo non-responders (n=10). Responders were defined as having at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at two years post-treatment.

Baseline levels of lipase and trypsin were lower, although not significantly, but improved to 115% of baseline in responders to immunotherapy six months after treatment. Non-responders and placebo subjects experienced a decline in lipase and trypsin to 80 - 90% of baseline during this time period. There were no differences in amylase levels between groups at baseline or six months after treatment. These preliminary findings suggest that lipase and trypsin may serve as biomarkers for response to immunotherapy in type 1 diabetes. As the authors note, further studies with larger participant numbers are needed to address this question.

In a review, Tas et al. explore whether metabolic dysfunction-associated steatotic liver disease (MASLD) may explain the increased risk of cardiovascular disease (CVD) in individuals with Type 1 Diabetes (T1D). The review manuscript focuses on observational studies, cohort studies, and meta-analyses that investigate the prevalence of MASLD in T1D populations and its association with CVD. Furthermore, the manuscript examines the physiological mechanisms that link MASLD and CVD, including

hepatic insulin resistance, systemic inflammation, and atherogenic dyslipidemia, to assess the independent contribution of MASLD to cardiovascular risk in T1D patients. The literature suggests that chronic inflammation and atherogenic lipid profiles associated with MASLD elevate the risk of CVD and recommends routine assessments of liver dysfunction in the care of patients with T1D to mitigate the risk of cardiovascular complications.

Chimatapu et al. report retrospective data from a group of 42 adolescent males followed at a single center who tested sufficient on the initial growth hormone stimulation test but continued to present with short stature or growth failure. Interestingly, 59% tested deficient upon reevaluation and were started on rhGH therapy, exhibiting an excellent response. The adult height was reported for half of the patients treated with rhGH who reached adult height, which was comparable to results reported for those with IGHD. The authors emphasize the importance of re-evaluating children who show ongoing evidence of inadequate growth despite previously normal growth hormone stimulation testing. They recommend longitudinal monitoring and retesting for patients who continue to experience growth failure and may benefit from rhGH therapy. The authors hypothesize that the evolving growth hormone deficiency (EGHD) is due to 'progressive decline or insufficient production of growth hormone (GH), especially during the period of pubertal development'.

In a case report of a 14-year-old male with insulinoma and primary hyperparathyroidism, a novel heterozygous mutation in MEN1 is reported and characterized. (Huang et al.) The mutation is also found in the proband's father, who exhibited only hyperparathyroidism in adulthood, suggesting that family members may present variations in clinical phenotypes. The grandparents and the father's siblings were unwilling to undergo genetic testing, opting only for screening of blood glucose, calcium, phosphate, and PTH, all of which were normal. No additional pathologic involvement of the pituitary gland, adrenal glands, or lungs was found in the proband. This manuscript underscores the importance of genetic testing in patients with MEN1 and their family members.

Rett syndrome (RTT) is an X-linked progressive neurodevelopmental disorder primarily affecting girls and is the second most common cause of genetic intellectual disability. RTT results in neurological regression between 6 and 18 months of age and is associated with varying degrees of neurological impairment. Recent data indicate that the endocrine system is frequently involved in RTT patients, including disorders of growth, bone health, the thyroid, pubertal onset, and weight abnormalities. However, systematic data on endocrinopathies in patients with RTT are limited. Pepe et al.'s systematic review manuscript aims to analyze the prevalence and types of endocrine comorbidities in the RTT population to facilitate early diagnosis and appropriate endocrinological management. Out of the 1090 studies screened, 22 met the inclusion criteria. The main endocrinopathies reported were malnutrition, bone abnormalities, and delayed puberty onset. The authors conclude that endocrinopathies are not uncommon in RTT patients and recommend screening and monitoring for endocrinopathies.

Digital health technologies are becoming an integral part of enhancing patient care and the management of chronic conditions. These technological advances can lead to increased adherence, a cost-effective healthcare system, and improved medication self-management. To assess the willingness of 22 healthcare providers to integrate the connected rhGH injection pen into their clinical practice, participatory workshops were conducted in Rome, Italy, and Seoul, Korea—two diverse healthcare ecosystems. (Rivera Romero et al.) This qualitative study explored current attitudes toward the digitalization of rhGH therapy through panel discussions, analyzed healthcare providers' perceptions regarding the potential acceptance of the connected device compared to other non-connected alternatives (e.g., pen and paper adherence diaries), and assessed factors affecting their intent to use and integrate digital health solutions that support rhGH therapy in clinical practice. The authors conclude that understanding the nuances of these perspectives is essential for developing strategies to address the challenges and capitalize on the opportunities presented by the ongoing digital transformation in healthcare. Although healthcare providers recognize the potential of digital health solutions to enhance patient engagement and, consequently, clinical outcomes, the participatory workshops highlighted several aspects of how this digital transformation is influencing treatment options and the necessity for digital literacy for successful implementation. (Rivera Romero et al.).

Finally, a manuscript by Zucchini et al. in the Study Group on Diabetes of the Italian Society of Pediatric Endocrinology and Diabetology (ISPED) is based on a systematic review of available scientific evidence and a Delphi consensus methodology, aiming to provide evidence-based recommendations for recognizing, risk stratifying, treating, and managing patients with hypoglycemia. The objective of these recommendations is to improve the timely recognition and prevention of hypoglycemic episodes and to apply the correct treatment, especially for patients using continuous glucose monitoring (CGM) or advanced hybrid closed-loop systems. Practical flow charts are included to aid clinical decision-making, which will be very helpful for clinicians, especially when caring for patients using CGM and other advanced technologies. Importantly, the authors explore the concept of 'fear of hypoglycemia' (FoH), nasal glucagon use, and educational support to fully address the needs of the Italian community.

Author contributions

MM: Writing – review & editing, Writing – original draft. SR: Writing – review & editing, Writing – original draft.

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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Endocrine features of Prader-Willi syndrome: a narrative review focusing on genotype-phenotype correlation

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Prader-Willi syndrome (PWS) is a complex genetic disorder caused by three different types of molecular genetic abnormalities. The most common defect is a deletion on the paternal 15q11-q13 chromosome, which is seen in about 60% of individuals. The next most common abnormality is maternal disomy 15, found in

around 35% of cases, and a defect in the imprinting center that controls the activity of certain genes on chromosome 15, seen in 1-3% of cases. Individuals with PWS typically experience issues with the hypothalamic-pituitary axis, leading to excessive hunger (hyperphagia), severe obesity, various endocrine disorders, and intellectual disability. Differences in physical and behavioral characteristics between patients with PWS due to deletion versus those with maternal disomy are discussed in literature. Patients with maternal disomy tend to have more frequent neurodevelopmental problems, such as autistic traits and behavioral issues, and generally have higher IQ levels compared to those with deletion of the critical PWS region. This has led us to review the pertinent literature to investigate the possibility of establishing connections between the genetic abnormalities and the endocrine disorders experienced by PWS patients, in order to develop more targeted diagnostic and treatment protocols. In this review, we will review the current state of clinical studies focusing on endocrine disorders in individuals with PWS patients, with a specific focus on the various genetic causes. We will look at topics such as neonatal anthropometry, thyroid issues, adrenal problems, hypogonadism, bone metabolism abnormalities, metabolic syndrome resulting from severe obesity caused by hyperphagia, deficiencies in the GH/IGF-1 axis, and the corresponding responses to treatment.

KEYWORDS

Prader-Willi syndrome (PWS), genotype-phenotype correlation, growth hormone (GH), metabolic syndrome, hypogonadism, bone metabolism, type 2 diabetes, thyroid

1 Introduction

Prader-Willi syndrome (PWS; MIM: 176270) is a rare and complex progressive disorder that affects multiple body systems, and is recognized as one of the most common causes of genetic obesity. It occurs in approximately 1 in 20,000-30,000 live births (1, 2), with an equal number of males and females affected.

1.1 Genetic background

Approximately 60% of patients with PWS have a deletion on the paternal chromosome 15q (del15q), located between common breakpoints (BPs – two proximal sites known as BP1 and BP2, and one distal site named BP3). There are two main classes of deletions: type I, which accounts for 40% of cases and is about 6 Mb in size spanning BP1-BP3, and type II, which makes up 60% of cases and spans 5.3 Mb between BP2 and BP3 (3) (Figure 1). In addition, around 8% of individuals with a deletion have a unique or atypical deletion size (i.e., not type I or II) due to various causes, such as unbalanced translocations (4).

Maternal uniparental disomy (mUPD), where both copies of chromosome 15 come from the mother, is responsible for approximately 35% of PWS cases and results from errors during meiosis. There are three types of mUPD: i) isodisomy, caused by errors in meiosis II leading to non-disjunction of sister chromatids followed by trisomy rescue; ii) heterodisomy, caused by errors in meiosis I with non-disjunction of homologous chromosomes and no crossing over; and iii) mixed UPD, related to errors in meiosis I with crossover events (3).

Finally, 1-3% of individuals with PWS, who have apparently normal chromosomes, have imprinting defects. PWS was the first human disorder to be linked to genomic imprinting (5). It occurs when genes inherited from the father on chromosome 15q11.2-q13 are not expressed, due to various genomic mechanisms (3). Most genes in this region are subject to genomic imprinting, meaning only the father's alleles are active while the mother's alleles are silenced through methylation.

This means that either the paternal chromosome has a maternal imprint-switch or the maternal chromosome has a paternal imprinting. This incorrect epigenotype is a result of a dysregulation in the imprinting mechanism in the parent's germ line, often caused by microdeletions in a critical region known as the PWS-imprinting center (6), a critical region involved in the regulation of the epigenetic modifications at this locus. In families affected by PWS due to a deletion in the imprinting center, the smallest overlapping region of deletion (PWS-SRO) is 4.3 kb and includes exon 1 of the SNRPN gene (7).

The region between BP1 and BP2 on chromosome 15q11.2-q13 contains four non-imprinted genes: NIPA1, NIPA2, CYF1P1, and

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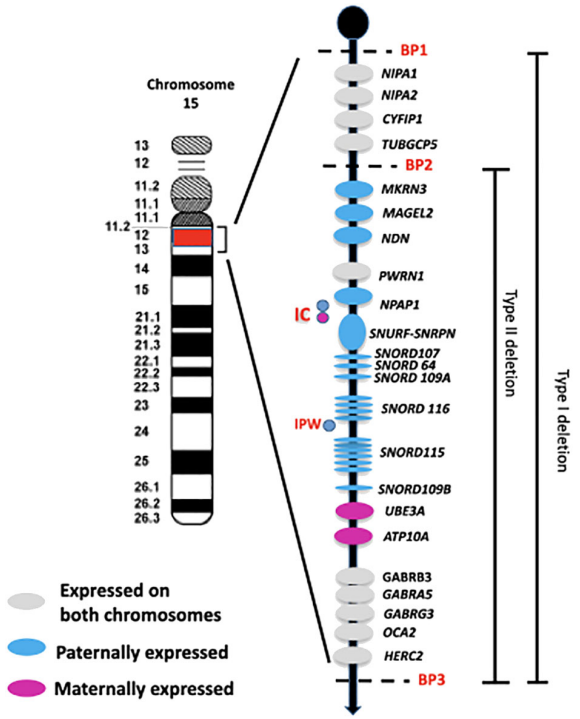

FIGURE 1
Schematic overview of the imprinted region 15q11q13. The paternally and maternally expressed genes are reported. The typical larger 15q11-q13 type I deletion involving breakpoints BP1 and BP3 and the typical smaller 15q1-q13 type II deletion involving breakpoints BP2 and BP3 are illustrated. IC: imprinting center controlling the activity of imprinted genes in the 15q11-q13 region. IPW: imprinted in Prader Willi syndrome. BP common breakpoint.

TUBGCP5 (Figure 1). A microdeletion of this 500 kb region does not cause PWS, but instead leads to a range of clinical manifestations, such as neurodevelopmental disorders that can include intellectual disability, speech impairment, and behavioral disorders. This collection of symptoms is collectively known as 'Burnside-Butler syndrome' (8), although not all individuals with this deletion show symptoms.

The PWS critical region is located between BP2-BP3 and contains genes that are crucially involved in growth processes, neurodevelopmental and hormonal regulation. These genes include *MKRN3*, *MAGEL2*, *NDN*, and the bicistronic *SNURF-SNRPN*, as well as C15orf2/NPAP1, a gene that is expressed from both alleles in the testis but only from the paternal allele in the brain (9).

The MKRN3 gene encodes a protein that belongs to a family of zinc finger proteins that act as an E3 ubiquitin ligase. MKRN3 seems to be involved in regulating the hypothalamic-pituitary-gonadal axis by inhibiting the release of gonadotropin-releasing hormone (GnRH) and contributing to the control of puberty onset (10). Pathogenic variants in MKRN3 can disrupt this regulatory function, leading to conditions such as central precocious puberty (CPP) (11). *MAGEL2* encodes the melanoma antigen L2, a member of the Melanoma Antigen Gene (MAGE) family of ubiquitin ligase regulators. It is highly expressed in the hypothalamus, where it plays a crucial role in the regulation of protein trafficking and recycling, which affect secretory granule biogenesis (12). Inactivating mutations in *MAGEL2* cause Schaaf-Yang syndrome (MIM: 615547), which is characterized by delayed psychomotor development, frequent intellectual disability, hypotonia, feeding difficulties, and various dysmorphic facial features.

The NDN gene encodes necdin, a member of the MAGE protein family that is mainly expressed in the brain. It is involved in the regulation of cell proliferation, differentiation, and survival, and is closely linked to the suppression of postmitotic neuron-specific growth. Additionally, it contributes to the regulation of neural stem cells, neuronal differentiation, and synaptic plasticity (13).

In this region, there is also a cluster of more than 80 C/D box small nucleolar RNA genes (snoRNAs) and several antisense transcripts. Within this cluster, SNORD116 is a cluster of genes that code for small nucleolar RNAs that are highly expressed postnatally in the hypothalamus, and are involved in regulating food intake and energy balance.

The correlation between genotype and phenotype in PWS remains unclear, as none of the genes in the PWS region have been specifically implicated in the syndrome on their own (6, 14). However, it is likely that these genes contribute to different extents to some aspects of the phenotype. Several studies suggest that SNORD116 snoRNA plays a key role in the cardinal PWS phenotype (3). While large deletions are the most common genetic defect in PWS, a few patients have been reported with microdeletions that specifically include the tandem array of 30 copies of *SNORD116*. Despite the function of *SNORD116* remaining elusive, these patients exhibit the main clinical features of PWS (15).

Thanks to advancements in molecular genetic testing, the diagnosis of PWS can now be confirmed very early in the neonatal period. The main genetic abnormalities - del15q, mUPD, and imprinting center defect - can all be identified using Comparative Genome Hybridization (aCGH) in combination with methylation-specific multiplex ligation-dependent probe amplification analysis (MS-MLPA). However, a high-resolution karyotype is still needed to detect cases with translocations or other rare chromosomal rearrangements involving chromosome 15 (16).

1.2 Endocrine features

It is important to note that PWS undergoes a complex evolution throughout an individual's lifespan. Typically, the characteristic clinical presentation becomes more apparent in adulthood (17, 18). In infants, major symptoms include severe muscle weakness, weak crying, lethargy, difficulty feeding leading to failure to thrive, as well as cryptorchidism in males and underdeveloped labia in females (19). During childhood, individuals with PWS display hyperphagia, a strong desire for food, and develop severe obesity along with various related complications such as impaired glucose metabolism, type 2 diabetes mellitus (T2DM), fatty liver, gallstones, dyslipidemia, hypertension, metabolic syndrome, and cardiovascular disease unless food intake is controlled (16, 20).

Hyperphagia in PWS is still not fully understood, and controlling appetite remains a challenge in managing these patients. However, the description of the six nutritional phases in PWS has significantly

improved our understanding of the natural history of the syndrome (21). Dysmorphic features such as a characteristic facial appearance, small hands and feet, and narrow hands with a straight ulnar border are common (5). Other manifestations include distinct behavioral profiles associated with intellectual disability, such as cognitive rigidity, behavioral outbursts, severe anxiety, skin-picking, and an increased risk of psychosis. Additionally, individuals with PWS may experience scoliosis and/or kyphosis, sleep abnormalities including both central and obstructive apnea, and multiple endocrine abnormalities (22). Particularly, endocrinopathies described in PWS include short stature and growth hormone deficiency (GHD), hypothyroidism, central adrenal insufficiency (CAI), isolated precocious pubarche, and hypogonadotropic and hypergonadotropic hypogonadism (16).

Clinical manifestations related to PWS appear to involve a complex dysregulation of the hypothalamus, affecting both the adeno- and neurohypophysis (23). The exact cause of this dysregulation is not well understood. It is important to recognize that the symptoms of PWS can vary in severity among individuals. Additionally, no specific genotype of PWS is linked to exclusive symptoms, but a diverse range of clinical phenotypes has been observed in patients with the main subtypes (del15q or mUPD) (3, 6).

2 Aim and search methods

Genotype-phenotype correlation is a relationship that examines how the presence of a clinical trait corresponds with a group of similar mutations. However, this correlation is reliant on accurate phenotyping. Studying genotype-phenotype correlations can offer insights into disease development, progression, severity, and activity, ultimately aiding clinicians in tailoring treatment plans and follow-up care. Pathways from genotype to phenotype in PWS are difficult to explain. So far, no genotype has been linked to any exclusive symptoms. The aim of this literature review is to describe and discuss the endocrine and metabolic issues underlying PWS, emphasizing potential genotype-phenotype correlations to assist healthcare professionals in their practice and to guide future research endeavors, in order to provide some benefit to people with the syndrome and their families.

Manuscripts were included after conducting a search on PubMed, Directory of Open Access Journals and Google Scholar using the keyword “Prader-Willi syndrome” and the keyword for each specific section (for instance “thyroid” or “growth hormone” etc.). Only manuscripts written in English were considered. We excluded review paper, and non-original research articles. Furthermore, additional papers were included if they were known to the authors of this review, even if they were not retrieved by the search. Finally, the references of relevant articles were screened.

3 Neonatal anthropometry

Delayed diagnosis of PWS may be partially due to a lack of timely recognition of perinatal and/or neonatal features. In fact, during the perinatal and neonatal period, PWS is often associated with a higher prevalence of labor induction, breech presentation, cesarean delivery, preterm or post-term delivery, low birthweight, and small for gestational age (SGA) newborns compared to the general pediatric population (19, 24). Pregnancy-related complications, such as polyhydramnios from reduced fetal swallowing causing uterine distension (19) and/or placental abnormalities due to the lack of beneficial influence of paternal genes (25, 26), have been suggested to explain preterm labor and SGA, respectively. Birth weight and weight-for-length of newborns with PWS are reduced by approximately 500 grams (27). Regarding birth length, the results from the literature are still inconclusive, with some studies reporting values comparable to healthy controls (26, 28), and others reporting values 1-2 cm shorter than normal (24, 27). Occipito-frontal circumference was found to be within normal ranges compared to the general population at birth (24, 27), as well as over the first year of life (29). A recent nationwide multicenter study provided growth charts of birth weight and length for gestational age specific for PWS (27), which may serve as a useful tool in supporting the suspicion of PWS in newborns.

To date, only a few studies have examined potential links between perinatal characteristics and the specific genotype of PWS, resulting in conflicting findings. The differences in the prevalence of preterm births among different genotypes remain uncertain, as some researches have reported similar rates between mUPD and del15q (24, 26, 30, 31), while others have found a higher prevalence in mUPD individuals (32, 33) or, conversely, in those carrying del15q (19). Additionally, mUPD has been linked to a greater likelihood of post-term births (32).

Most studies have not identified differences in birth weight among specific genotype groups (26, 27, 31, 34). However, Dudley et al. discovered lower birth weight only in the del15q group, irrespective of gender (28). On the other hand, the prevalence of SGA was significantly higher in mUPD carriers compared to patients with del15q or imprinting center defects, with a prevalence rate of 31%, roughly twice as high as in the other two genotype groups (24). Furthermore, several studies have indicated a correlation between parental weight (28) or maternal age (24, 26) and birth weight specifically in PWS patients with mUPD or imprinting center defects.

Regarding birth length, a large cohort study (27) found a significant decrease only in females with del15q compared to those with mUPD, with no clear genotype-phenotype correlation observed for male neonatal size. These results contradict those reported by Whittington et al. (26), who found lower birth length values only in patients with mUPD or imprinting center defects, who were also shorter than patients with the del15q.

4 Thyroid function

Thyroid function in PWS has been extensively studied, and, like other endocrine disorders, its cause is believed to be centrally located in origin due to a dysregulated hypothalamic-pituitary-thyroid (HPT) axis.

In PWS, central hypothyroidism (CH) is the most common thyroid function abnormality, with a prevalence ranging from 2% to

72.2% globally (35, 36) and varying age of onset (Supplementary Table 1). Screening for hypothyroidism in newborns with PWS often produces negative results, as their levels of TSH and total T4 are typically within the normal range and comparable to those of healthy individuals (37). However, there have been a few cases of congenital hypothyroidism in PWS newborns due to an ectopic thyroid gland (38, 39).

Congenital hypothyroidism appears to be more common in infants and toddlers with PWS, with the highest incidence in the 1 to 3-year-old group, gradually decreasing over time (40), likely due to a temporary dysfunction of the hypothalamic-pituitary-thyroid axis (41, 42). During this phase, treatment with levothyroxine may be necessary if there is a simultaneous decrease in free T4 (fT4) and free T3 (fT3) levels, or an increase in TSH levels, to prevent significant impacts on intellectual development (43, 44). In later stages of life, thyroid function may normalize, even in individuals who were previously treated with levothyroxine (44). It is recommended that adults with PWS undergo regular and periodic screening of fT4 and TSH levels, as untreated hypothyroidism can have adverse effects on metabolic rate, body mass index, and cardiovascular health (45).

The influence of sex on thyroid status and whether genotype could be associated with a different pattern of thyroid function still remains unclear. Regarding genotype, the prevalence of CH in children with PWS was not significantly different among the paternal deletion, mUPD, and unclassified groups (40). Similarly, no relationship between hypothyroidism and genotype was found in a cohort of 122 adults with PWS (45). Recent studies have shown that there are no significant differences in the prevalence of CH between the sexes (42), although previous works observed that CH was more common in males with PWS (41).

The relationship between thyroid function and recombinant human (rhGH) therapy, particularly the influence of rhGH on serum thyroid hormone levels and increased conversion of thyroxine (T4) to triiodothyronine (T3), has been evaluated in some studies (41, 46). RhGH therapy does not seem to increase the prevalence of different forms of hypothyroidism in PWS (41, 46), but it is important to evaluate thyroid hormones before, during, and after rhGH treatment to confirm euthyroidism and prevent the possible risks of previously unmasked and/or untreated hypothyroidism.

The autoimmune cause of impaired thyroid function has never been described or studied in patients with PWS. Thyroid autoantibodies are rarely measured in PWS patients, likely due to the well-known central origin of hypothyroidism. Only one study, which evaluated 21 PWS children, reported a case of mild positivity for anti-thyroid peroxidase antibodies (39). However, the possibility of autoimmune thyroid involvement could be considered, given the recently proposed autoimmune cause of pituitary dysfunction associated with PWS (47, 48). Further research is needed to clarify this issue.

Regarding hyperthyroidism, a single case with an unclear origin treated with thiamazole was reported in a group of adult patients with PWS (45). It is important to regularly monitor the thyroid function of PWS patients at different stages of life, taking into account factors such as levothyroxine treatment, rhGH therapy, and the emergence of new symptoms of hypothyroidism.

Based on the data available in the literature, there does not appear to be a clear correlation between genotype and thyroid dysfunction in individuals with PWS. Yang et al. (42) found that infants with PWS caused by mUPD had higher TSH levels compared to those with PWS caused by a paternal 15q11-q13 deletion, even though the TSH levels were still within the normal range. Additionally, there were no differences in fT3 and fT4 levels between these two groups. Conversely, in adults with PWS caused by a 15q11-q13 deletion, TSH levels within the normal range were higher than in PWS individuals with mUPD. Again, no differences in fT3 and fT4 levels were found between the groups (45). Therefore, further evaluation will be necessary to determine if there is a possible correlation between genotype and the different patterns of thyroid dysfunction.

5 Adrenal gland function

Disturbances in the hypothalamus-pituitary-adrenal (HPA) axis, with an inadequate response during stressful conditions like infections or episodes of dehydration, have been suggested as potential causes of unexpected and unexplained deaths in PWS. This theory is supported by the discovery of adrenal atrophy upon autopsy (49) and reduced cell numbers in the hypothalamic paraventricular nuclei (50).

CAI in PWS is not well understood, as studies have reported varying results (ranging from 0% to 60%) using different dynamic tests, such as the insulin tolerance test (ITT), metyrapone test (MT), glucagon test (GT), and the currently most used low-dose and standard dose Synacthen stimulation test (LDSST and SDSST) (Table 1) (51–62).

Based on early data that show a high prevalence of CAI (56, 56), some authors have suggested that all patients with PWS should be treated empirically with hydrocortisone during stressful situations unless CAI has been ruled out. The high prevalence reported in these studies, however, could be related to the use of ACTH levels for the diagnosis of CAI, rather than 11-deoxycortisol, and the high cut-off used (33 pmol/l) (54, 57, 61). More recent studies, using 11-deoxycortisol or a lower cut-off of ACTH after MT or other dynamic tests, reported a much lower prevalence (52–55, 57–62). Based on this low CAI prevalence, several authors advised against routine administration of hydrocortisone during psychological stress, illness, or surgery (61) to avoid excessive treatment. Additionally, reports of adrenal crisis during surgery are currently only anecdotal (63). Other reports by Oto et al. (55) and Grootjen et al. (62) have shown a delayed peak of cortisol after ITT, ACTH and 11-deoxycortisol after MT.

The literature data generally supports the evidence that individuals with PWS in common clinical practice do not typically experience adrenal insufficiency, are not usually treated with daily glucocorticoids, and do not generally experience adrenal crises during surgery or other stressful procedures and events. Currently, there is no consensus among endocrinologists on the best diagnostic approach, management, or need for empiric glucocorticoid treatment during critical illness or before surgery.

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TABLE 1 Summary of the studies with stimulation tests for central adrenal insufficiency in PWS.

Author, year	Subjects involved	Methods (test, normal values)	Diagnosis of CAI	Genotype-phenotype correlation
De Lind Van Wijngaarden, 2008 (51)	25 children	MT (ACTH ≥ 33 pmol/l)	15 (60%)	No significant difference in genotype between patients with and without CAI
Corrias, 2012 (52)	84 children	LDSST, followed by confirmatory SDSST (cortisol > 500 nmol/l, for SDSST also an increase in cortisol of ≥ 250 nmol/l)	4 (4.8%)	In patients with del15q the cortisol peak was significantly lower than in mUPD cases; mUPD genetic subclass resulted a predictor of peak response
Grugni, 2013 (53)	53 adults	LDSST, followed by confirmatory SDSST (cortisol > 500 nmol/l)	4 (7.5%)	Genetic abnormality was not predictive of CAI
Obrynba, 2018 (54)	21 patients (4-53 years)	LDSST (cortisol ≥ 427.6 nmol/l) followed by MT (11-deoxycortisol ≥ 200 nmol/l)	0	No statistically significant difference between del15q and mUPD in LDSST results
Oto, 2018 (55)	36 children	ITT (cortisol > 18.1 μg/dl - 499.3 nmol/l- or increase in cortisol by ≥ 9.1 μg/dl - 251 nmol/l)	0	In PWS subjects with del15q peak levels of cortisol after ITT were significantly delayed compared to mUPD patients

CAI, central adrenal insufficiency; del15q, deletion of chromosome 15; ITT, insulin tolerance test; LDSST, low-dose and standard dose Synacthen stimulation test; MT, metyrapone test; mUPD, maternal uniparental disomy; SDSST, standard dose Synacthen stimulation test.

Regarding the genotype-phenotype correlation, it is currently not possible to define a clear correlation, as evaluated by a small number of authors so far. de Lind van Wijngaarden et al. (51) found no significant differences in genotype between pediatric patients with and without CAI diagnosed by MT, while Corrias et al. (52) reported a significantly lower cortisol peak after LDSST in children with del15q compared to mUPD, a genetic subclass identified as a predictor of the peak response. However, this result with LDSST was not confirmed by Obrynba et al. in children (54) or by Grugni et al. in adults (53). Oto et al. (55) did indicate a delayed cortisol peak response after ITT in del15q compared to mUPD.

Regarding basal cortisol, children with PWS are generally able to produce adequate levels in daily life, as demonstrated by studies showing normal average morning basal serum cortisol (64–66) or salivary cortisol (51). Lower values of morning basal cortisol, although within the normal range compared to the general population, were reported in a large cohort of PWS children by Angulo et al. (64), but not by Beaulove et al. (60) and Oto et al. (55), nor by Butler et al. in a population of adults and children with PWS compared to a control group with non-syndromic obesity (65). Angulo et al. and Butler et al. (64, 65) found no correlation between morning basal cortisol and specific genetic subtypes. Based on the literature review, primary adrenal insufficiency (PAI) has not been reported in PWS patients.

Another issue regarding adrenal function in PWS is the presence of premature pubarche, reported in the literature with a very variable prevalence, from 14% to 60% (66–73). Siemensma et al. (67) and Gaston et al. (70) found no significant differences in the proportion of patients with premature pubarche in subjects with different genetic subtypes. Increased plasma levels of DHEA-S have been reported in children with PWS compared to the healthy pediatric population, with no associations between DHEA-S levels and BMI or percentage of fat (66–68). Finally, Siemensma et al. (67) showed no significant differences in DHEA-S levels between genetic subtypes in children, while Gaston et al. (70) reported the highest DHEA-S levels in patients with del15q.

6 Bone metabolism

Individuals with PWS typically have a bone structure characterized by short stature, low bone mineral density (BMD), and low bone mineral content (BMC) (74), often leading to orthopedic issues such as scoliosis, kyphosis, hip dysplasia, flat feet, and genu valgum (75, 76).

The process of bone formation is continuous throughout life, and the acquisition of bone minerals in early childhood is crucial for achieving optimal BMD levels in late adolescence. Bone health is influenced by the interaction between osteoblasts (bone forming cells, OBs) and osteoclasts (bone resorbing cells, OCs), which are primarily regulated by the Wnt/β-catenin pathway and the receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) axis, respectively (74).

In childhood, individuals with PWS often have normal BMD levels when adjusted for their reduced height (77), but during adolescence, they may experience a decrease in total BMD and BMC. As a result, it is estimated that 29% to 44% of adults with PWS may suffer from bone fractures due to the higher prevalence of osteoporosis, reported to be as high as 21% (78).

The factors contributing to bone impairment in individuals with PWS are not fully understood, but possible explanations include loss of function of genes in the q11-q13 region of the paternal copy of chromosome 15, reduced production of sex hormones during puberty, as well as a relative GHD during childhood and adolescence (5). Additionally, low calcium intake, insufficient levels of vitamin D, lack of physical activity, and changes in serum adipokines may also play a role in bone impairment in these individuals (79).

Regarding the genes in the PWS critical domain that are involved in skeletal abnormalities, deletion of Snord116 has been shown to have negative effects specifically on the bone cortical compartment (80). Deletion of *MAGEL2* is linked to Schaaf-Yang syndrome, which is characterized by a unique skeletal phenotype with abnormal BMD due to increased OCs activity and enhanced

transformation of OBs into adipocytes (81). *MAGEL2* deletion is also associated with decreased levels of N-oleoyl serine, which has a positive correlation with BMD and OBs activity (82).

Hypogonadism is a common feature in individuals with PWS. This condition is a well-recognized risk factor for osteoporosis, and it is important to start hormone replacement therapy (HRT) in a timely manner to prevent bone loss (78). A cohort study of 22 PWS patients showed a significant improvement in BMD and lean body mass after starting HRT (83). Another study found that individuals with PWS who received HRT had higher BMD compared to those who were not treated (84). Therefore, it is recommended that individuals with PWS start HRT as soon as hypogonadism is identified.

There is limited data on the effects of rhGH treatment on BMD in individuals with PWS. A study by Bakker et al. (85) found that total body BMD and lumbar spine BMD remained stable in prepubertal PWS children during 9 years of GH therapy, but decreased during adolescence due to incomplete pubertal development, emphasizing the importance of prompt replacement therapy. In a group of adult PWS subjects, GH treatment for 2 years did not effectively improve low bone mass (86). However, there are conflicting findings on the effects of GH therapy on BMD in PWS, as some studies have shown that GH administration in PWS adults has a positive effect on bone mineralization and geometry (87). Nakamura et al. (88) reported a low BMD in 61.5% of subjects with PWS, with an improvement in lumbar BMD Z-score after more than 4 years of GH administration. Additionally, a higher incidence of osteoporosis was found among PWS adults who did not receive GH therapy during adolescence (88).

Data on vitamin D levels in individuals with PWS are still limited and inconclusive. Panfili et al. (89) studied a group of pediatric PWS patients and did not find any significant differences between PWS children and the control group, although there was a slight decrease in obese PWS individuals compared to those of normal weight. In contrast, Barrea et al. discovered that adults with PWS had lower levels of vitamin D compared to the control group, regardless of differences in body fat (90).

Brunetti et al. demonstrated the involvement of the Wnt/β-catenin and RANK/RANKL/(OPG) axis in PWS by showing high levels of RANK-L and low levels of OPG in both children and adults with PWS, with a bias towards RANK-L (91). Additionally, sclerostin levels were significantly higher in children and lower in adults with PWS compared to controls, indicating that RANK-L, OPG, and sclerostin may play a crucial role in regulating bone turnover in individuals with PWS.

Recently, irisin, a myokine secreted by the muscle, has sparked great interest due to its involvement in bone, adipose tissue, and brain homeostasis. Specifically, in young mice, irisin injection mimicked the effects of exercise by increasing cortical bone mass and strength (92). Hirsch et al. found higher amounts of salivary irisin in obese individuals with PWS compared to non-obese controls, while plasma levels of irisin did not show significant changes between the two groups (93, 94). Faienza et al. demonstrated that irisin serum levels in PWS patients were similar to those of controls (95), however, both pediatric and adult PWS subjects with del15q had lower irisin levels compared to controls, whereas no difference was observed between PWS subjects with mUPD and healthy controls.

LIGHT/TNFSF14 is a cytokine produced by immune cells that affects both fat and bone metabolism; Faienza et al. showed that serum LIGHT levels were significantly higher in both PWS children and PWS adults than in controls (96). Additionally, LIGHT levels were found to have a negative correlation with DEXA parameters. LIGHT serum levels were influenced by various factors including vitamin D and DXA parameters related to bone and fat quality, indicating the important role of LIGHT as a marker of bone impairment.

Table 2 summarizes the most important studies about bone metabolism in PWS.

7 Growth hormone - insulin-like growth hormone -1 axis and growth

Impaired growth is a common feature of PWS, attributed to a lack of GH/IGF-1 and a lack of pubertal growth spurt. Infants with PWS also show other clinical signs of GHD, such as low muscle tone, small hands and feet, increased body fat percentage, and reduced lean mass.

GHD is documented in 40-100% of children with PWS, although the severity can vary from mild to severe (98–100). Without rhGH replacement therapy, the average final height for men is reported to be 155-160 cm, and for women, it is 145-150 cm (101).

The cause of impaired GH secretion in PWS is still being debated, mainly because early childhood-onset obesity is so common in this population. It is widely recognized that obese individuals who are otherwise healthy tend to have lower GH secretion compared to lean individuals. Obesity typically leads to changes in the IGF system, resulting in lower IGF-1 availability. However, most studies show that the total measured circulating IGF-1 levels in obese individuals are normal or even elevated (102). In individuals with PWS, IGF-1 levels are often reported to be low in many studies, regardless of BMI, indicating a GHD. Additionally, levels of IGFBP-3, a protein that binds to IGF-1, are also found to be low in PWS (99, 103, 104). Unfortunately, research on the IGF system in PWS has been limited overall.

The GH response to growth hormone-releasing hormone (GHRH) stimulation test with simultaneous administration of pyridostigmine in obese children showed increased GH levels, attributed to the reduced induced somatostatinergic tone. Meanwhile, children with PWS still exhibited lower GH response and reduced serum IGF-1 concentrations, confirming a genuine GHD (105). GHD is a progressive process in individuals with PWS. According to Cohen et al., stimulated levels of GH in infants with PWS were higher compared to older children or adults, suggesting that younger children may have a normal GH pituitary reserve (106). Additionally, some studies have indicated that in young children with PWS, standard stimulation tests (clonidine and arginine) result in low GH peaks in most patients, whereas combined stimulation tests (GHRH+arginine or pyridostigmine) show normal results in the majority of PWS tested children. This

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TABLE 2 Summary of the studies about bone metabolism in PWS.

Author, year	Objective	Subjects	Results
Bakker NE, 2015 (85)	To determine effects of long-term GH treatment and puberty on BMD of total body and lumbar spine	77 children with PWS who remained prepubertal during GH treatment for 4 years and 64 children with PWS who received GH treatment for 9 years participated in the study	Total body BMD and lumbar spine BMD remain stable in prepubertal PWS children but decreases during adolescence, due to the incomplete pubertal development
Jørgensen AP, 2013 (86)	To investigate bone mass in a group of adult PWS subjects and study the effects of GH treatment on BMD	46 adults with PWS were randomized to GH or placebo for 12 months, followed by open prospective GH for 24 additional months	No changes in BMD were observed with continuous GH treatment for 24 months
Longhi S, 2015 (87)	To evaluate bone geometry, density and strength in a group of adult obese patients with PWS and to examine the effect on bone of treatment with GH and sex steroids	41 adults with PWS treated with GH and 46 healthy controls	GH treatment improves bone geometry but not bone density. Bone strength was significantly reduced in PWS patients who did not receive GH and had been treated with sex steroids.
Nakamura Y, 2014 (97)	To investigate BMD in PWS patients and to verify the efficacy of and scoliosis deterioration with GH administration for osteoporosis	148 PWS (141 treated with GH) patients who underwent lumbar spine BMD testing	61.5% of subjects had low BMD; GH administration significantly improved the lumbar BMD
Panfili FM, 2023 (89)	To analyze 25OHD levels in pediatric PWS patients in comparison with a control group	192 children and adolescents with PWS and 192 healthy controls	No statistically significant differences in 25OHD levels were observed between the PWS population and the controls; a slightly decrease was observed in obese than normal weight PWS
Barrea L, 2020 (90)	To investigate 25OHD levels and the dietary vitamin D intake in PWS adults	15 PWS adults and 15 control subjects	PWS had lower 25OHD levels than in the control group, regardless of body fat differences
Brunetti G, 2018 (91)	To test the hypothesis that the levels of bone remodeling mediators (RANKL, OPG, sclerostin, DKK-1 serum levels, and bone metabolism markers) may be altered in PWS subjects	12 PWS children, 14 PWS adults and 31 healthy controls	High RANKL and low OPG serum levels were found both in children and adults; sclerostin serum levels were significantly higher in children and lower in adults than controls
Faienza MF, 2021 (95)	to determine circulating levels of irisin in children and adult PWS patients	78 subjects with PWS (26 children and 52 adults) and 80 healthy controls (26 children and 54 adults)	Irisin serum levels in PWS patients did not differ when compared with controls but both pediatric and adult PWS with del15q displayed lower irisin levels than controls while no difference was observed between PWS subjects with mUPD and healthy controls; in pediatric PWS the vitamin D levels affected irisin serum concentration
Faienza MF, 2023 (96)	To evaluate LIGHT serum levels and to identify correlations with parameters of bone and fat metabolism	8 children and 52 adult PWS patients compared to age and sex-matched controls	Serum LIGHT levels were significantly higher in both PWS children and in PWS adults than controls; LIGHT was also negatively correlated with DEXA

BMD, bone mineral density; del15q, deletion of chromosome 15; DEXA, dual-energy x-ray absorptiometry; GH, growth hormone; mUPD, maternal uniparental disomy; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; OPG, osteoprotegerin; 25OHD, 25-hydroxy vitamin D.

suggests that very young PWS children may have impaired hypothalamic GHRH secretion with a normal GH pituitary reserve, which could diminish as they grow older, possibly resulting in insufficient levels of GH (107). Therefore, the efficacy of standard GH stimulation tests as an indicator of GH status in younger individuals with PWS is still a topic of debate. Additionally, it has been demonstrated that, using the potentiated GHRH+arginine stimulation test, GH response is greater in children than in adults (108).

Reduced GH bioactivity has also been shown in children and adolescents with PWS. This should be taken into account in the small number of affected individuals with PWS and growth failure who exhibit normal growth hormone responses after standard stimulation tests (109). Additionally, low growth hormone secretion has been found in 24-hour frequent blood sampling studies, which is consistent with neurosecretory GHD based on current knowledge (108).

Furthermore, impaired GH secretion in these individuals is linked to a reduction in visceral fat and elevated ghrelin concentrations relative to the degree of obesity (110). Ghrelin, a stomach-derived hormone that stimulates GH secretion, can be increased in individuals with PWS before the onset of obesity, which is a distinct feature not typically seen in common obesity or other genetic obesities (111, 112). However, due to the various forms of ghrelin and their effects, it is currently challenging to establish clear relationships with GH secretion in PWS (113).

During the transition phase, around 20% of individuals with PWS displayed a GHD status based on BMI-dependent criteria

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(114), while GHD was found in 8-38% of adult patients (115). Some genotype-phenotype correlations have been identified in relation to GH secretion (116), but there is a lack of literature on the changes in the IGF system (Table 3). It is worth noting that patients with mUPD are more likely to have GHD compared to those with deletions (80% versus 25%) (100, 107–109). No significant differences have been observed between individuals with type I and type II deletions (98, 107, 108, 117). Additionally, only those with paternal deletions show a correlation between GH response to stimulation and BMI, which is not seen in subjects with mUPD (108). Patients with paternal gene microdeletions also exhibit better GH responses to stimulation than those with mUPD, who experience delayed GH peaks after stimulation as a distinctive feature. Interestingly, children with del15q11-q13 are longer at birth than those with mUPD, and the latter are shorter than PWS patients without deletions (105). Deletions in PWS, particularly those affecting genes involved in neural development, brain function, infertility, and circadian rhythm, such as SNORD116, can result in proconvertase 1 deficiency leading to GHD, short stature, hypogonadism, hyperghrelinemia, relative hypoinsulinemia, and T2DM. Therefore, special attention should be paid to these children for early replacement treatment consideration (100).

In both the transition phase and in adult patients, the GH peak response to GHRH+arginine was significantly higher in patients with del15q than in those with mUPD (114, 117).

TABLE 3 Observed genotype-phenotype correlations in Prader Willi Syndrome related with GH secretion.

Author, year	GH secretion in PWS	Genotype-Phenotype correlations
Di Giorgio G, 2014; Marostica E, 2013; Alves C, 2020; Grugni G, 2011 (100, 107–109)	Correlation between different PWS genotypes and incidence of GHD	Patients with mUPD have a higher incidence of GHD than those with deletion while no differences have been reported between individuals with both type I and type II deletions
Marostica E, 2013 (108)	Correlation between GHD and BMI in PWS genotypes	A correlation between the GH response to stimulation and BMI has been demonstrated only for individuals with paternal deletion
Di Giorgio G, 2014 (107)	Response to stimulation tests differs in different PWS genotypes	Patients with paternal gene microdeletions present better GH responses to stimulation tests with respect to PWS patients with mUPD
Grugni G, 2021 (114)	Prevalence of GHD in PWS during the transition phase	Patients with del15q had higher GH peak response to GHRH+arginine compared with subjects with mUPD
Grugni G, 2011 (117)	Comparison of stimulated GH response in adults with PWS due to different genetic subtypes	Subjects with mUPD had a lower mean peak GH response and integrated GH secretion than those observed in patients with del15q

BMI, body mass index; del15q, deletion of chromosome 15; GH, growth hormone; GHD, growth hormone deficiency; GHRH, growth hormone-releasing hormone; mUPD, maternal uniparental disomy.

8 GH therapy

GH therapy in children with PWS was initially approved by the Food and Drug Administration in 2000 and by the European Medicines Agency in 2001. The Consensus Guidelines of the Growth Hormone Research Society in 2013 recommended GH therapy for all children with genetically confirmed PWS, in addition to dietary, lifestyle, and environmental interventions (118).

The goals of GH therapy in PWS go beyond simply improving growth (99), as it has a positive impact on various clinical aspects and the quality of life of affected individuals. GH therapy results in improved body composition, with a decrease in fat mass and an increase in lean mass (119, 120). It also has a positive effect on muscle strength, motor function (121, 122), and bone mineral density (123). Furthermore, improvements in neurological and behavioral impairments, such as achieving motor milestones earlier and developing adaptive skills, have been reported (124).

In adulthood, GH treatment has been shown to have beneficial effects on lean and fat mass, muscle performance, and quality of life (116). The positive effects on body composition and fat distribution are maintained for an average of 17 years, regardless of GH secretory status (125). However, it is necessary to confirm the presence of GHD after reaching final height before initiating GH therapy (118).

According to previous expert opinions (118, 119), in pediatric age, it is not necessary to assess GH/IGF-1 function before starting GH treatment. However, some contraindications to GH therapy are present in subjects with PWS, such as severe obesity or severe respiratory impairment, acute critical illness, uncontrolled diabetes, active cancer, and active psychosis (118). There is no consensus on the age at which to start therapy, but there is agreement on starting before the onset of obesity, which often occurs at around 2 years of age (118). Some studies have shown a better benefit in starting therapy in the first year of life, between 4 and 6 months (122, 124) or even at 3 months (118). The recommended dose is 1.0 mg/m2 per day, achieved within approximately 3-6 months of starting treatment (118). This dose is higher than that usually used in congenital GHD, but lower doses showed a reduced effect on body composition (126, 127). If body surface area is not used for dose calculation, it is recommended to use a nonobese weight for height as a reference in obese patients (118). PWS patients seem to be particularly sensitive to GH, as at standard doses they often present IGF-1 levels above the normal limits, in particular in individuals with PWS due to mUPD (128); special care must be taken in biochemical monitoring for a possible association between IGF-1 values and the occurrence of adverse effects (129–131). Therefore, it is recommended that IGF-1 levels remain within the upper normal range (+1 to +2 SDS) for age-matched normal children during GH therapy, and it is indicated in infants and children with PWS to start therapy with a daily dose of 0.5 mg/m2 per day to minimize side effects, with subsequent adjustments toward 1.0 mg/m2 per day (117). Scheermeyer et al. reported that treating infants with a low dose of GH (4.5 mg/m2/week) leads to a normalization of IGF-1 and height SDS within the first year of therapy with minimal risk of side effects (132). However, no relationship between malignancies and GH treatment has been reported in a large cohort of patients with PWS (133).

A recent study has shown that the genetic diagnosis of PWS and the start of GH therapy occur earlier in patients with a del15q than in those with mUPD and an imprinting center defect (ID) (31). According to the study, patients with del15q are typically diagnosed in the second year of life, while other PWS patients are diagnosed in the fourth year of life. Additionally, GH therapy is initiated at an average age of 4.24 years in del15q patients, 7.3 years in mUPD 15, and 6.42 years in those with mUPD/ID. Although mean IGF-1 values before GH treatment were within the reference range for age and sex, del15q patients had significantly higher IGF-1 levels compared to mUPD/ID (-0.83 ± 0.46 SDS vs -1.03 ± 0.43 SDS), despite no significant differences in height SDS.

Among patients with mUPD, GHD is more common and severe than patients with a del15q (116). However, Oto and colleagues found that there was no significant difference in yearly height improvement between these two groups of patients (134). Nonetheless, when looking at subgroups meeting the criteria for GHD, mUPD patients showed a significantly better response to rhGH treatment in terms of annual growth rate than deletion patients (0.42 ± 0.26 for del15q vs 0.7 ± 0.21 for mUPD; p=0.0044). Variability in response to GH therapy may also be linked to the presence of an exon-3 deletion polymorphism of the GH receptor (d3 allele), which is present in around 50% of the Caucasian population (135). In children with PWS, those who are heterozygous or homozygous for the d3 allele are more sensitive and responsive to GH treatment, leading to a significant increase in growth compared to those who are homozygous for the full-length GHR allele (136). Similar outcomes are observed in non-PWS children undergoing GH therapy (135).

According to literature, adult patients with del15q show higher body weight and BMI compared to those with mUPD. However, both groups have similar proportions of fat mass, adipocyte volume, and insulin resistance markers (137). These findings suggest that growth hormone therapy during childhood and/or adolescence may lead to a better metabolic profile in adults with PWS who have del15q, but not in those with mUPD, thus lessening the phenotypic impact of the deletion.

In terms of intellectual disability, Butler et al. analyzed cognitive skills and proposed that rhGH therapy could help prevent cognitive decline in individuals with PWS, especially those with mUPD (138).

Differences between mUPD and del15q have also been documented in relation to psychiatric issues: individuals with del15q exhibit higher levels of aggression compared to those with mUPD (p=0.007) (139), are more likely to engage in skin picking (p=0.008) (139) and are more prone to developing compulsions and self-injury (140, 141). On the other hand, mUPD 15 is associated with an increased risk of anxiety disorders (p=0.04) and psychoses (142). Moreover, GH therapy has shown a significant link to the development of anxiety and delusions (2.7 times increased association with anxiety, p=0.05; 14 times increased association with delusions, p=0.03). Specifically, GH therapy poses a higher risk of anxiety in individuals with mUPD (3.25-fold increase) compared to those with del15q (2.73-fold increase), regardless of treatment duration (139).

In relation to orthopedic issues, a higher incidence of scoliosis has been reported among individuals with PWS who have received rhGH treatment and have a deletion (p=0.011). On the other hand, scoliosis is more common in patients with mUPD who have not undergone therapy (p=0.039) (143). However, data from the literature do not show any significant connection between scoliosis and rhGH treatment (144). The same authors also noted that kyphosis occurs in similar proportions in individuals with a deletion and mUPD undergoing rhGH therapy, but among untreated patients, it is more prevalent in those with a deletion rather than mUPD (p=0.001). Additionally, they found differing associations between genotype and phenotype in individuals with a deletion receiving rhGH treatment compared to those with mUPD, particularly in terms of having a flat back of the head (p=0.002) and abdominal stretch marks (p=0.006), which were more common in the former group. They also observed that ocular signs, such as eyes that slant downwards and strabismus, were more frequent in individuals with mUPD receiving growth hormone therapy (p=0.006 and p=0.011).

9 Obesity

The clinical symptoms of PWS can vary depending on age. In later childhood, patients can become severely obese unless their food intake is closely monitored by family and caregivers. Individuals with PWS often experience insatiable hunger, known as hyperphagia, and other compulsive behaviors, typically developing between the ages of 2 and 5 years (21, 145, 146). While the exact cause of hyperphagia in PWS is not fully understood, it is believed to involve abnormalities in hypothalamic satiety pathways. This may include hypothalamic malformations or related neurochemical mechanisms (147, 148).

However, abnormal eating behaviors in PWS cannot be solely attributed to hypothalamic dysfunction. Functional brain imaging techniques, such as functional magnetic resonance imaging (fMRI) and fluoro-deoxy-glucose positron emission tomography (FDG-PET), have shown that the orbitofrontal cortex (OFC) is activated when individuals consume food until they feel full. Signals related to satiety and visceral cues within the OFC significantly influence food perception, resulting in a response that reflects the food's reward value or appeal. Individuals with PWS have been found to have altered OFC responses to satiety in functional brain imaging studies (149). The olfactory system also plays a crucial role in nutrition and social behavior, with connections to the endocrine regulation of energy balance (150). It can trigger a specific appetite for relevant foods, known as sensory-specific appetite, helping us detect and respond to food in our environment. A study involving adults with PWS (150) revealed abnormal brain reward system activity, particularly at the right amygdala level, in response to food odors. This heightened amygdala activity corresponds to the rapid and craving-inducing reaction to the odor, aligning with the concept of food-related addictive behaviors. Additionally, this increased activity is linked to the severity of hyperphagia, suggesting that changes in the brain circuits processing food odors may contribute to this phenomenon in PWS (150). Individuals with PWS have elevated levels of ghrelin and oxytocin, both of which are involved in addictive behaviors. These hormones not only impact the

hypothalamus and limbic system but also influence the olfactory bulb, affecting sensory processing. Elevated fasting levels of ghrelin, hyperadiponectinemia, hypoinsulinemia, and increased ghrelin/PYY values compared to obese controls have been described (151). Ghrelin abnormalities may contribute to hyperphagia and food-related thoughts and cravings in PWS, while oxytocin dysfunction may affect emotional processing and appetite regulation (150).

None of the genetic defects associated with PWS are directly linked to specific clinical traits. However, some connections have been found between different molecular classes and clinical features, including eating behavior and obesity.

Several studies have investigated possible differences in compulsive behavior among the three most prevalent genetic subtypes (Type I deletion, Type II deletion, and mUPD). These behaviors often center around food-related problems, like searching for and saving food, acting on impulse, and repeatedly asking for food. These behaviors ultimately play a role in the onset of obesity.

Therefore, it is clear that individuals with mUPD show a lower frequency and severity of compulsions compared to those with deletions. However, the available data on potential differences between the two types of deletions are inconclusive (145). Type I deletions, which affect a greater number of genes, seem to be associated with more severe compulsions than Type II deletions (145). Yet, other studies have found no noticeable distinctions (152, 153).

Dykens et al. (153) conducted a cohort study with eighty-eight individuals (43 males and 45 females) with PWS ranging from 5 to 51 years old. Their research did not find any clear differences between deletion subtypes, but they did notice significant variations within each subtype in terms of the relationship between age and behavior. Specifically, in the Type I group, they found negative correlations between age and behavior, possibly due to non-imprinted genes like CYFIP1. The CYFIP1 gene, which is also involved in other developmental disorders like 15q abnormalities, may contribute to age-related changes in the phenotype of individuals with Type I PWS due to its haploinsufficiency in those cases.

Deficiencies in imprinted genes, such as MKRN3, MAGEL2, and/or NDN, are not enough on their own to cause the full range of symptoms seen in individuals with PWS. However, they do seem to be involved in the development of overeating and obesity.

There is evidence to suggest that mutations in the MAGEL2 gene may contribute to the characteristic weight gain seen in individuals with PWS. Overeating is linked to a malfunction in the hypothalamic arcuate nucleus, which is where neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), and leptin interact to regulate food intake and body weight. NPY and AgRP stimulate food intake, while POMC works to suppress it. In experimental models, a loss of MAGEL2 expression disrupts the normal response of POMC neurons to leptin, leading to increased food intake and uncontrolled fat storage (154, 155). A study by Schaaf et al. identified specific point mutations in the paternal allele of MAGEL2 in four individuals with PWS, where weight gain was a prominent feature along with muscle weakness, developmental delays, and hypogonadism (81).

Kanber and colleagues described a patient with deletions in MKRN3, MAGEL2, and NDN, exhibiting only obesity, developmental delay, and a high pain threshold as the primary clinical criteria for PWS (14). Regarding the series of long non-coding RNAs (lncRNAs) which can be involved in the regulation of gene expression at transcriptional and post-transcriptional levels in PWS, within the long SNURF-SNRPN transcript, there are a series of Small Nucleolar RNAs (snoRNAs) thought to participate in DNA methylation, alternative splicing, and post-transcriptional regulation, such as SNORD116 and SNORD115.

The SNORD116 cluster is crucial in the PWS phenotype. As reported, SNORD116 is involved in controlling NPY neuronal functions, and thus food intake and energy homeostasis. Experimental models on Snord116-KO mice showed PWS features such as hyperphagia (156–159). De Smith et al. (160) reported a 19-year-old male with hyperphagia and severe obesity, mild intellectual disability, and hypogonadism with a 187 kb deletion that included SNORD116.

The SNORD115 gene contains a complementary sequence of 18 nucleotides that matches the mRNA for the serotonin receptor 5-HT2C. Studies on 5-HT2C receptor knockout mice have shown that lack of SNORD115 protein may cause problems with the 5-HT2C receptor, leading to abnormal eating behavior and late-onset obesity (161, 162). Additionally, aside from SNURF-SNRPN and the SNORD gene family, the potential role of NPAP1 in obesity development should be considered (162). Kanber et al. found two patients with deletions in NPAP1, SNURF-SNRPN, and the SNORD genes, who exhibited major clinical signs of PWS, including obesity (14).

In summary, although data linking genotype and obesity in human studies is limited, these findings underscore the importance of taking a comprehensive approach to studying phenotypes throughout life.

10 Metabolic syndrome

The metabolic syndrome (MetS) is a common complication of overweight/obesity, leading to T2DM and cardiovascular disease. Diagnosis requires meeting at least 3 of the following criteria: central obesity, arterial hypertension, high triglyceride levels, altered glucose metabolism, and low HDL cholesterol levels (163). Insulin resistance (IR) and obesity are thought to be central factors in the development of MetS (163, 164). Identifying predictive factors for MetS may improve treatment outcomes and prevent severe complications.

Individuals with PWS appear to have a healthier metabolic profile compared to those with essential obesity. This is due to their distribution of subcutaneous fat, higher insulin sensitivity, increased levels of adiponectin and HDL cholesterol, lower rates of fatty liver disease, and reduced cytokines (165–170). However, PWS patients may have higher blood glucose and blood pressure levels, with no significant differences in insulin or insulin resistance compared to controls (171, 172). Additionally, PWS individuals are more likely to have altered glucose metabolism and T2DM (173), although the exact mechanisms are still unknown. The prevalence

of MetS in PWS adults and children is 34% and 7% respectively (172, 174), potentially contributing to the reported early mortality rates in the PWS population (171, 175).

Several studies have compared non-syndromic obesity and PWS, but there is limited data available on the clinical features associated with specific PWS genotypes. In particular, information on the genotype-phenotype characterization of MertS and its characteristics is fragmented.

It has been found that adipocyte size is increased in PWS compared to obese controls, with variations observed in different genotypic subclasses (176). Patients with a del15q genotype tend to have higher BMI (137, 176, 177) and HbA1c levels, despite similar values of glycemia, insulinemia, insulin resistance, body composition, metabolic profile, adipocyte size, resting energy expenditure, hyperphagia score, and levels of ghrelin compared to those with mUPD, suggesting a possible association between mUPD genotype and a more adverse metabolic profile, even when adjusted for BMI. The most common form of dyslipidemia in PWS patients, occurring in about 50% of cases, appears to be a decrease in HDL cholesterol, with no significant difference between genetic subtypes (137). Additionally, data from a study involving 108 PWS subjects indicated that the del15q group had a lower risk for low HDL cholesterol and a trend towards a lower risk for MetS compared to non-deleted patients (172). These findings suggest that the del15q genotype group may have the healthiest metabolic profile among adult PWS individuals.

Talebizadeh et al. (168) assessed the body fat composition, IR, leptin, and lipid profile in 55 individuals with PWS and 18 obese controls. They found that in obese individuals, there was a direct correlation between insulin levels and weight, as well as between BMI and subcutaneous fat area. However, these correlations were not observed in the PWS group. On the other hand, PWS patients showed a strong direct correlation between insulin levels and fat area (both visceral and subcutaneous). Specifically, visceral fat and insulin levels were significantly related in the del15q group. Additionally, visceral fat area and triglycerides were directly correlated with the age of individuals in the obese control group but not in the PWS groups. In the del15q and mUPD groups, a positive correlation was found between glucose and triglyceride levels. In the del15q group, there was also a positive correlation between cholesterol and glucose, triglycerides and visceral fat area, insulin and visceral fat area, insulin and triglycerides, and insulin and total cholesterol. These findings suggest a different and specific metabolic profile, at least for one genetic subtype.

Adiponectin is a hormone that sensitizes insulin, and low levels of adiponectin are associated with obesity and MetS. Patients with PWS have higher levels of adiponectin compared to obese individuals, regardless of their physical characteristics (178). This was shown in some recent studies (169, 174, 179) involving different numbers of PWS patients. These studies found no statistical differences in parameters such as insulin, glucose, HOMA, triglycerides, HDL, BMI, and blood pressure among different PWS genotypes.

Irisin regulates glucose levels and insulin sensitivity by promoting glucose uptake and glycogenolysis, as well as reducing gluconeogenesis. It may have a role in obesity and MetS through controlling body weight and regulating the accumulation of white adipose tissue. In a study comparing 25 PWS patients and 25 obese individuals, irisin levels were significantly lower in PWS patients with a del15q compared to obese controls, while patients with a mUPD did not show significant differences compared to controls (180) (Table 4).

11 Glucose metabolism

Obesity plays an important role in the risk of T2DM in individuals with PWS, as in the general population (171, 173, 181). However, the prevalence of T2DM in PWSs tends to be lower than in obese controls (165, 182). The relationship between adipose tissue (AT) and glucose metabolism in PWS remains largely unknown, although several studies have observed indices of lower IR compared to controls (166, 183, 184). The possible explanations for the higher insulin sensitivity in PWS could be the relatively lower visceral adipose tissue (VAT) and higher subcutaneous adipose tissue (SAT) than BMI-matched controls, as well as the predominant accumulation of subcutaneous fat in the trunk and proximal limbs, with a lower ratio of trunk-to-appendicular fat (167, 185, 186); the downregulation of many IR-associated genes in the AT of subjects with PWS, including those encoding proinflammatory markers (176); the elevated levels of ghrelin (187) and adiponectin (183); the impaired GH secretion (110); the lower high-sensitivity C-reactive protein and IL-6 concentrations (166); the different composition of the gut microbiota compared to patients with obesity (188). Therefore, the pathophysiological mechanisms underlying T2DM in PWS might be different from those involved in common obesity. Since IR is an important risk factor for the development of T2DM, the relatively lower IR could be a protective factor against the risk of developing T2DM in PWS patients, which is common in adults but rarely develops in childhood. More generally, the prevalence of T2DM in adults with PWS ranges from 11 to 25%, compared to 5 to 7% in the general population (173, 189), while it is reported less than 2% in patients younger than 18 years of age (190). On the other hand, data from a cohort of 74 children with PWS showed the presence of impaired glucose tolerance by OGTT in 4% of subjects, but no T2DM (36). In this context, the different genotypes (del15q or mUPD) do not appear to be correlated with the development of altered glucose homeostasis in PWS (174). Furthermore, no genotype-phenotype correlation studies seem to emerge from the literature review. In addition, it should be noted that rare cases of T1DM have also been described (191).

The average age at diagnosis of T2DM in PWSs is about 20 years (171, 173, 191). The onset of T2DM is closely related to the presence of obesity in PWS, the latter related to an insufficient and delayed satiety response, due to uncontrolled food intake mechanisms caused by a hypothalamic dysfunction (21, 192). Currently, the only available control for hyperphagia for most people with PWS is external supervision by mentors with constant supervision, which is challenging and distressing for both caregivers and patients. Access to food must be limited to ensure low caloric intake, considering that children with PWS

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TABLE 4 Summary of the studies about MetS in PWS.

Author, year	Study design and purpose	Study population	Outcomes	Further considerations
Talebizadeh, 2005 (168)	Cross-sectional study; To examine difference in metabolic profile between PWS vs OC subjects	37 PWS: 20 del15q, age 22.4 ± 7.4 y, 17 mUPD, age 24.2 ± 9.7 y, 18 OC: 25.9 ± 13.3 y,	Positive correlation in PWS del15q group between: - Cholesterol and glucose; - Triglyceride and VFA; - VFA and insulin; - Insulin and triglyceride; - Insulin and total cholesterol. Positive correlation in PWS del15q and mUPD group between glucose and triglyceride;	possible different VFA regulation between PWS and simple obesity subjects. Lower Insulin resistance in PWS.
Kennedy, 2006 (169)	Cross-sectional study; To examine diabetes and cardiovascular risk in PWS subjects compared with OC subjects.	20 PWS: 13 del15q, 7 mUPD; age: 27.7 ± 10.3. 14 OC: age: 26.9 ± 11.4 y	No relevant differences in adiponectin and other variables between PWS del15q and PWS mUPD subjects.	Higher adiponectin levels and less insulin resistance (proportionate to the obesity status) in PWS compared with OC subjects
Brambilla, 2011 (174)	Cross-sectional study; To estimate the frequency of MetS and its components in PWS pediatric subjects	109 PWS (50 obese and 59 non-obese): 58 del15q, 31 mUPD, 20 MET+; age: obese 11 ± 8.1 y; non-obese 10,5 ± 6 y 4 T2DM; 69 GHt. 96 OC: age: 10 ± 5.8 y	No differences in evaluated parameters between PWS genotypes.	Low MetS frequency in non-obese PWS. Similar MetS frequency in obese PWS and OC
Grugni, 2013 (172)	Cross-sectional study; To estimate the occurrence of MetS and its components in PWS adult subjects	108 PWS (87 obese and 21 non-obese): 73 del15q; 27 mUPD; 2 TRAS; 6 MET+ age: obese 26 ± 9 y; non-obese 21 ± 9 y. 23 T2DM 85 OC: age: 28 ± 8 y	Lower risk in PWS del15q for low HDL Lower MetS risk in PWS del15q among genotypes	healthier MetS parameters in non-obese PWS compared with obese PWS and OC.
Lacroix, 2015 (176)	Cross-sectional study; To compare several MetS and obesity parameters between PWS and OC subjects	42 PWS: 27 del15q; 14 mUPD; 1 IMPRINTING MUTATION. (34 MET+ excluded from analysis); age: 25.5 ± 8.9 y; 10 T2DM 42 OC age: 27.5 ± 9.0 y	Lower BMI in PWS mUPD compared with PWS del15q. No differences in the body compositions or metabolic phenotypes or Adipocyte volume between the genotypes (thus, higher relative values in PWS mUPD normalized for BMI). No difference in transcriptomic signature between the genotypes	Lower trunk FM and better metabolic profile in PWS. Significantly higher adipocyte size in PWS vs OCs.
Coupaye, 2016 (137)	Observational study; To compare body composition and metabolic profile between PWS genotype subgroup.	73 PWS: 47 del15q 26.5 ± 9.9 y; 27 mUPD 23.7 ± 6.9 y; Age: 25.5 ± 8.9 y; 14 T2DM	Significantly higher BMI in del15q subjects Significantly higher HbA1c in del15q subjects without diabetes, compared with mUPD patients. No differences in endocrine profile between del15q and mUPD. Higher proportion of percent body fat, adipocyte size and insulin resistance (related to BMI) in UPD compared with del15q. Lower BMI, percentage of body fat and adipocyte size in del15q subjects treated with GH, compared with mUPD	. Decreased HDL as the most frequent type of dyslipidaemia, with no difference between genetic subtypes
Laurier, 2015 (177)	Retrospective study; To analyze medical, psychosocial and social features of PWS subjects according to gender, age, and genotype.	154 PWS: 101 del15q, 24 mUPD, 3 IMPRINTIG DEFECT, 23 MET+, 3 TRAS; age: 28.4.	Significantly higher BMI in del15q group compared with non-del15q group.	

(Continued)

TABLE 4 Continued

Author, year	Study design and purpose	Study population	Outcomes	Further considerations
Marzullo, 2020 (179)	Cross-sectional study To evaluate MetS parameters, serum ureic acids and its metabolic effect in PWS subjects compared with OCs.	89 PWS: 67 del15q, 21 mUPD, 1 MET+; age: 28.4 ± 8.7 y 180 OC age: 29.8 ± 7.5 y	No differences in the evaluated parameters between PWS genotypes.	Lower serum uremic acid levels in PWS compared with OC.
Mai, 2022 (180)	Cross sectional study To explore the role of circulating irisin in relation to the metabolic profile and body composition in obese pediatric population with and without PWS	25 PWS: 15 del15q, 10 mUPD. age: 11.1 ± 0.6 y 25 OC age: 12.6 ± 0.7 y	significantly lower irisin levels in del15q group, compared with OC.	Lower irisin levels in PWS compared with OC, Strong association between irisin with insulin resistance, C-peptide and insulin OGTT 120' levels.

BMI, body mass index; del15q, deletion of chromosome 15; FM, Fat mass; GHt, GH treatment; GIP, glucose-dependent insulinotropic polypeptide; GLP1, glucagon-like peptide-1; HbA1c, glycosylated hemoglobin; HDL, High density lipoprotein; HOMA, homeostasis model assessment; hs-CRP, high sensitivity C-reactive protein; MET+, positive methylation test, cryptogenic; MetS, Metabolic syndrome; mUPD, maternal uniparental dysomy; OC, obese control; OGTT, Oral Glucose Tolerance Test; QUICKI, quantitative insulin sensitivity check index; SFA, Subcutaneous fat area; TRAS, de novo translocation involving chromosome 15; T2DM, type 2 diabetes mellitus; VFA, visceral fat area; y, years.

require 20-30% less energy intake than healthy children of the same age (193). Healthy lifestyles are at the forefront of management of altered glucose metabolism in PWS, with metformin as the first-line drug therapy. When monotherapy is not sufficient, sulfonylureas, meglitinides, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors, glucagon-like peptide-1 (GLP-1) agonists (liraglutide, exenatide, semaglutide, dulaglutide) or insulin can be combined (171). In addition, sodium-glucose cotransporter-2 (SGLT-2) inhibitors have been used in adult patients with PWS in uncontrolled situations (194). Taking into consideration the pediatric age, only insulin and metformin are approved for children (over 10 years of age), but recently GLP-1 receptor agonists (liraglutide) have been introduced for the treatment of T2DM. Moreover, liraglutide has been approved for use in obese non-diabetic children older than 12 years. Some studies have reported the beneficial effects of incretin mimetics as an effective therapy for hyperphagia and obesity in PWS, considering its potential effects on ghrelin suppression, central appetite suppression (through its action on pro-opiomelanocortin POMC/CART neurons and cocaine- and amphetamine-regulated transcripts in the arcuate nucleus (195),), increased energy expenditure (EE) and stimulation of insulin secretion, which could counterbalance the hypoinsulinemia reported in PWS (196–200). However, the data are still conflicting and other authors have reported no effect of liraglutide on weight loss either in children and adolescents with PWS (201).

The recommendations for screening for T2DM and metabolic syndrome are like the general guidelines for subjects with obesity (119). Patients with PWS should be screened for hemoglobin A1C, lipid profile, and transaminases at baseline and then annually. Regarding common diabetes-related complications, there are surprisingly few reports in the literature (202), and their follow-up is identical to that used in the general population.

Although new drugs such as GLP-1 agonists show potential in controlling weight, appetite, and blood glucose in subjects with PWS, diet, physical activity, and behavioral modifications remain the main strategies to promote good metabolic health in these patients.

12 Puberty and fertility in females

PWS is characterized by dysfunction of the hypothalamus, leading to multiple endocrine disorders, including hypogonadism (203, 204). In female patients, the clinical manifestation of hypogonadism can vary widely and may change at different stages of life (204, 205). While newborns with PWS often have underdeveloped clitoris and labia minora, puberty and breast development typically start on time. However, the progression of puberty is slow, and incomplete development is commonly seen later in life. Most patients experience primary amenorrhea, but some may have spontaneous menarche at a much older age (around 20 years), followed by secondary amenorrhea or irregular periods (73, 203, 204). Overall, the prevalence of hypogonadism in adult females with PWS ranges from 54 to 100% (73, 203, 204).

Hypogonadism in females with PWS is believed to be primarily caused by dysfunction of the hypothalamus. However, more recent studies suggest that primary ovarian dysfunction also plays a role (206, 207). In some patients, a combination of central and peripheral hypogonadism may be present, making them unique from individuals with more typical disorders (207, 208). Some authors have reported extremely low or undetectable inhibin B levels alongside normal or subnormal Anti Mullerian Hormone (AMH) levels, indicating a specific defect in folliculogenesis. However, a small subgroup of women have detectable inhibin B levels, potentially indicating partial ovarian follicular development and serving as a possible marker of fertility (205–208). Pregnancy has been documented in a small number of women with genetically confirmed PWS (204, 209, 210). Additionally, precocious puberty has rarely been observed in females with PWS (211). MKRN3, a gene located in the critical region of PWS, has been implicated in regulating pubertal development in typical individuals. MKRN3

deficiency has been identified as a common genetic cause of CPP, with different prevalence rates in sporadic (0.5-17-5%) versus familial (9-46%) cases (212, 213). Research on the influence of MKRN3 on pubertal development in females with PWS is limited.

Mariani et al. (214) evaluated MKRN3 protein levels in 80 individuals with PWS at various stages of pubertal development. They found that MKRN3 levels were measurable in 49 patients with PWS, but did not correlate with phenotype, genotype, or gonadotropin levels. This suggests that different genetic mechanisms at the tissue level may be involved in pubertal development in individuals with PWS (213, 214).

Many studies have investigated the potential correlation between genetic background and hypogonadal dysfunction in individuals with PWS, but no data have shown an impact of the various genetic defects (such as 15q11-13 paternal deletion, mUPD, or imprinting defect) on pubertal development and fertility (73, 203, 204). Despite a lack of connections between PWS genotype and puberty in humans, research on animal models replicating PWS has suggested that certain genes may be involved in hypothalamic-ovary dysfunction.

The human chromosome 15q11-q13, also known as mouse chromosome 7C, is an imprinting domain regulated by bipartite imprinting centers (IC). Studies on this chromosome region in the PWS mouse model have revealed varying expression and methylation in different tissues (215). Specifically, research by Mapendano et al. (216) has demonstrated abundant expression of the IC transcript in the brain and ovaries of the mouse model, particularly in granulosa cells of developing oocytes. In regards to the Necdin gene, which has been proposed as a potential candidate for PWS in humans due to its lack of expression in the brain, the mouse knockout model exhibited impaired development of GnRH neurons (217). Taken together, these preliminary findings from experimental models suggest a potential influence of gene expression and methylation at the brain and oocyte/follicle levels on pubertal development in individuals with PWS.

13 Puberty and fertility in males

PWS presents many challenges, with hypogonadism being identified as the most common hormonal deficiency among affected individuals. Studies by Partsch et al., Matsuyama et al., and Pellikaan et al. have reported that the prevalence of hypogonadism in adult males with PWS ranges from 57% to 100% (218–220). Driscoll et al. (221) have emphasized the significant impact of hypogonadism in PWS, leading to issues such as genital hypoplasia, incomplete pubertal development, and infertility in most cases.

The development of hypogonadism in males with PWS is diverse, with a variety of causes from central to peripheral hypogonadism. Hirsch et al. and Radicioni et al. have highlighted the occurrence of peripheral hypogonadism, a combination of central and peripheral forms, and pure hypothalamic deficit in some cases (206, 222). Interestingly, there is no established correlation between genotype and phenotype, as reported by Lecka-Ambroziack and Pellikaan et al. (71, 220).

Hypogonadism affects males with PWS of all ages. In infancy, it is common to observe cryptorchidism, scrotal hypoplasia, and a short penile length (175). Eiholzer et al. recommend human chorionic gonadotropin (hCG) treatment for males with PWS and cryptorchidism, as it can lead to lower testes and improved genital size before urologic surgery (223). Interestingly, cryptorchidism does not seem to be linked to gonadotropin levels, and there is no correlation between the age at orchidopexy and inhibin B levels, suggesting cryptorchidism does not play a major role in hypergonadotropic hypogonadism (203).

During puberty, males with PWS commonly experience incomplete and delayed development, alongside frequent precocious adrenarche. Besides hypothalamic dysfunction, primary testicular involvement (especially in the seminiferous epithelium) and moderate Leydig cell dysfunction all contribute to abnormal pubertal development in males with PWS (224, 225). Testicular histology often shows reduced or absent spermatogonia, with a progressive degeneration of germ cells during puberty (226). Lecka-Ambroziack, Linnemann et al., Siemensma et al. discuss the intricacies of pubertal development in PWS, with premature adrenarche seen in around 30% of cases. This could potentially lead to early puberty and compromised adult height without treatment (22, 67, 72, 227). In males with PWS, premature pubarche is associated with early pubic hair growth, elevated levels of serum dehydroepiandrosterone sulfate or serum dehydroepiandrosterone, and advanced bone age. These factors might reduce the positive effects of growth hormone on adult height (67).

While hypogonadism is the most common change in the pituitary-gonadal axis, CPP, though rare, has been documented in children with PWS and could be linked to loss-of-function variations in the MKRN3 gene at the 15q11-q13 locus (228, 229). The MKRN3 gene is widely recognized as a cause of CPP in the general population (230). Normally, *MKRN3* is believed to prevent the start of puberty before adolescence at high levels in the brain. A deletion of the MKRN3 allele from the father in PWS can lead to early puberty. Alternatively, high levels of 17-hydroxyprogesterone and DHEA sulfate were found in patients with CPP, suggesting elevated adrenal androgen levels as previously reported in PWS. Therefore, a combination of a father-derived MKRN3 allele deletion, basal adrenarche, and hypothalamic-pituitary acceleration may play a role in the early onset of precocious puberty (229). The type of hypogonadism - hypogonadotropic, hypergonadotropic, or a mix of these - only becomes apparent in late adolescence or early adulthood and appears to stabilize after the age of 20 years (225).

14 Discussion

PWS is a disorder caused by the lack of expression of certain genes on chromosome 15. This can be due to different genetic mechanisms, such as deletion of the paternal chromosome 15q11-q13, maternal uniparental disomy for chromosome 15, abnormalities of the imprinting center, or translocations involving chromosome 15. The main characteristic of PWS is hypothalamic dysfunction, which leads to overeating, body temperature instability, increased pain

tolerance, and pituitary issues resulting in various endocrine disorders. Autoimmunity could contribute, at least in part, to the hypothalamus-pituitary axis impairment in these individuals, in addition to the genetically determined dysfunction (47). However, we cannot exclude that anti-pituitary antibodies could be an "epiphenomenon".

Researchers have looked into whether the symptoms of PWS differ based on the underlying genetic cause. It has been observed that individuals with PWS due to deletion often experience sleep problems and speech difficulties (231) more frequently, with those with type I deletion displaying more compulsive behaviors and performing better academically than those with type II deletion (232, 233). In contrast, individuals with mUPD tend to have more neurodevelopmental impairments, including autistic features and behavioral issues, but typically demonstrate higher IQ levels compared to those with deletion of the critical region of chromosome 15 (16, 153, 232–234). These findings suggest that different genetic backgrounds contribute to the variation in symptoms, especially when it comes to neurodevelopmental aspects.

Prompted by this evidence, we reviewed the literature on endocrine features in PWS to evaluate whether a genotype-phenotype correlation can be established. We considered all the endocrine disorders described in this syndrome and concluded that the available data do not support such a correlation, except for some minor features.

Literature data indicate that PWS patients have lower birth weight and a higher prevalence of preterm delivery and SGA (24) compared to the general population, and a tendency for post-term birth (32). Lower birth weight has been inconsistently described in newborns carrying the 15q11.2-q13 deletion (26–28, 31, 34). Whether perinatal characteristics are specific to genotype remains unclear, and further research is needed to clarify this issue, irrespective of pregnancy management.

Thyroid disorders are quite common in these individuals, and the dysregulation of the hypothalamic-pituitary-thyroid axis is the most frequent cause (35–45). Studies that have attempted to find a correlation between different genotypes and phenotypes have been unsuccessful, suggesting that monitoring thyroid function should be done at any age (Supplementary Table 1).

Similar to the thyroid gland, the adrenal gland may also produce insufficient cortisol, with the most common cause being central dysfunction. This can result in primary and secondary ACTH deficiency, especially during stressful situations. Cortisol levels were found to be lower in children with PWS due to del15q compared to those with UPD (52), but this was not consistent with other data from children (54) and adults (55). There was no correlation between genotype and fasting cortisol levels (64, 65), although DHEA-S levels were found to be higher in individuals with del15q, leading to a higher rate of premature adrenarche in the paper by Gaston et al. (70) but not in the paper by Siemensma et al. (67). It is important to note that the diagnostic tests used to assess adrenal function are not consistent across studies, which may explain some discrepancies in findings. Fortunately, individuals with PWS typically do not experience adrenal crisis and do not require glucocorticoid treatment. There is still ongoing debate about the use of hydrocortisone for critical illness and surgery in these individuals, with no consensus reached yet.

Growth mechanisms are considered as one of the most important research fields in PWS, both because of short stature and the availability of a treatment for over 20 years now (100, 107–109). GHD has a 3 times higher incidence in patients with mUPD than in those with del15q. These latter patients also show a correlation between GH response to standard stimulation tests and BMI, as well as a better GH response to standard stimulation compared to mUPD patients (108). Current studies on IGF binding-protein cleavage and IGF bioavailability could offer new insights into the physiopathology of the GH-IGF-1 axis and linear growth mechanisms (235). In individuals with mUPD or ID, GH therapy appears to prevent cognitive decline, suggesting that cognitive skills may be more impaired in these individuals due to more severe GH secretion insufficiency.

Currently, growth hormone therapy is discontinued when adolescents reach adulthood. In a recent letter to the editor, Höybye et al., on behalf of the Clinical and Scientific Advisory Board of The International Prader-Willi Syndrome Organisation (IPSWO), proposed that growth hormone treatment should be extended to adults with genetically confirmed PWS as well. This is an intriguing proposal, as it is widely recognized that in adulthood, growth hormone has positive effects on maintaining normal metabolism and body composition, physical fitness, and beneficial effects on cardiovascular risk factors, and ultimately, quality of life in these individuals (236).

The absence of the sense of satiety is a key characteristic of PWS, resulting in excessive eating and weight gain, leading to MetS and T2DM. Although there is no evidence supporting a correlation between genotype and body fat distribution or obesity, research has suggested that certain genetic mechanisms involving the MKRN3 and MAGEL2 genes, as well as Small Nucleolar RNAs, may play a role in controlling body weight (14, 81, 156–162). While these genes are likely involved in the development of excessive eating and obesity in individuals with PWS, none of them fully explain the entire PWS phenotype.

Obesity is the initial stage leading to MetS and T2DM. Despite being incomplete (Table 4), the available data on genotype-phenotype correlation suggests that individuals with PWS who have the del15q genetic subtype often have a higher BMI compared to other genetic subtypes. However, they also tend to have a healthier metabolic profile, which reduces the risk of complications from MetS (133, 176, 177). Additionally, compulsive eating behavior appears to be more common in individuals with mUPD (144), while those with the del15q subtype seem to respond better to multidisciplinary rehabilitation interventions (180) due to a lower prevalence of psychiatric disorders (5, 237, 238).

Various studies have shown no differences in MetS parameters among different genetic subtypes within large population studies (169, 174, 179). This implies that the regulation of body weight and the development of MetS are more likely due to psychiatric, behavioral, and developmental factors rather than solely genetics. Surprisingly, the incidence of T2DM in PWS individuals is lower

than expected (165, 182), indicating they have a healthier metabolic profile compared to weight-matched healthy individuals.

Further investigation into the food-seeking behavior leading to severe obesity could reveal distinctions between individuals with mUPD and del15q, aiding researchers in understanding the extent to which metabolic disorders stem from neurodevelopmental issues or genotype-phenotype correlation. This information can help predict complications and guide early interventions for obesity.

Data regarding the onset of puberty and fertility are inconsistent, and a growing amount of data is emphasizing the importance of new genes in controlling puberty. It can be said that there is no clear link between the genes identified in the critical region of PWS and delayed puberty and/or hypogonadism. Additional studies, whether *in vitro* or using animal models, as well as pre-clinical and clinical studies involving humans, would be beneficial in further understanding this form of hypogonadism.

15 Conclusions

In conclusion, the available data on endocrine disorders associated with PWS suggest that there is no clear genotype-phenotype association. Only some features of the GH-IGF-1 axis appear to be dependent on the different genotypes. However, a limitation of the analyzed papers is that the cohorts are not homogeneous, which could partially account for the contrasting results from different papers. Another potential limitation in drawing conclusions about genotype-phenotype associations is the small number of patients recruited in many of the referenced studies. Original research based on a very large cohort of people living with PWS, aiming to investigate the genotype-phenotype correlation, would be appreciated as they would shed new light on these features, supporting clinicians in optimizing follow-up.

Author contributions

SM: Writing – review & editing, Writing – original draft. LZ: Writing – review & editing, Writing – original draft. SV: Writing – review & editing, Writing – original draft. VC: Writing – original draft, Supervision, Data curation. AC: Writing – original draft, Supervision, Data curation. LD: Writing – original draft, Supervision, Data curation. MF: Writing – original draft, Supervision, Data curation. DF: Writing – original draft, Supervision, Data curation. LG: Writing – original draft, Supervision, Data curation. ML: Writing – original draft, Supervision, Data curation. EM: Writing – original draft, Supervision, Data curation. RP: Writing – original draft, Supervision, Data curation. ES: Writing – original draft, Supervision, Data curation. MS: Writing – original draft, Supervision, Data curation. MW: Writing – original draft, Supervision, Data curation. SB: Writing – original draft, Supervision, Data curation. CB: Writing – original draft, Supervision, Data curation. RB: Writing – original draft, Supervision, Data curation. MC: Writing – original draft, Supervision, Data curation. DC: Writing – original draft, Supervision, Data curation. FD: Writing – original draft, Supervision, Data curation. RF: Writing – original draft, Supervision, Data curation. NF: Writing – original draft, Supervision, Data curation. NI: Writing – original draft, Supervision, Data curation. LM: Writing – original draft, Supervision, Data curation. CM: Writing – original draft, Supervision, Data curation. VR: Writing – original draft, Supervision, Data curation. CS: Writing – original draft, Supervision, Data curation. GF: Writing – original draft, Resources, Investigation, Funding acquisition. LI: Writing – original draft, Supervision, Data curation. VS: Writing – original draft, Supervision, Funding acquisition, Data curation. AS: Writing – review & editing, Supervision, Project administration, Conceptualization. MG: Writing – review & editing, Supervision, Project administration, Conceptualization. GG: Writing – review & editing, Supervision, Project administration, Investigation. MD: Supervision, Data curation, Conceptualization, Writing – review & editing, Writing – original draft.

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Supplementary material

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Evolving growth hormone deficiency: proof of concept

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Introduction: We present the evolution of GHD in adolescent males with persistent growth failure, in whom the diagnosis was established after a second GH stimulation test (GST).

Methods: We performed a retrospective chart review of children who presented for short stature (height less < 2SD for mean/mid-parental height) and/or growth failure (sustained growth velocity < 0 SD) to pediatric endocrinology at Mount Sinai Kravis Children's Hospital, New York and who had 2 GSTs. Data collected from electronic medical records were analyzed using SPSS v28.0

Results: Of 53 patients included, 42 were males. Average GH peak on initial GST was 15.48 ± 4.92 ng/ml, at 10.07 ± 2.65 years, mean height -1.68 ± 0.56SD(28% had <2SD), IGF-1 -1.00 ± 0.88SD. After 2.23 ± 1.22 years, at 12.04 ± 2.41years, height SDs decreased to -1.82 ± 0.63SD and IGF-1 was -1.08 ± 0.84SD. At repeat GST, average GH peak was 7.59 ± 2.12 ng/dL, with 36% ≤7 ng/dl and 32% in puberty. 12 males reached adult height of 0.08 ± 0.69 SD with a mean height gain of 1.83 ± 0.56SD($p<0.005$), IGF-1 of -1.15 ± 0.81SD after 4.64 ± 1.4 years of GH.

Conclusion: We offer evidence for Evolving Growth Hormone Deficiency (EGHD) through repeat GST in children with persistent growth slowdown, even with pubertal progression; emphasizing the need for careful longitudinal follow-up to make accurate diagnosis.

KEYWORDS

growth, growth hormone stimulation test, growth hormone deficiency, idiopathic short stature, growth hormone therapy

Introduction

Growth hormone deficiency (GHD) is a common endocrinological cause of growth failure and short stature with a reported incidence of 1/4,000 to 1/10,000 children (1). GHD can be congenital or acquired and be present in combination with other pituitary deficiencies or as an isolated defect. The diagnosis of isolated, acquired GHD (IGHD), which represents the majority of cases, can be challenging (2). The GH Research Society consensus statement recommends that IGHD diagnosis should include auxological, biochemical, and radiographic evaluations (1, 3–5). In children with clinical criteria for GHD, growth hormone stimulation tests (GST) continue to play a key role in the diagnosis despite the known limitations of GST (6–9). A peak growth hormone (GH) level below 10 μg/dL is largely still clinically considered the cut-off for the diagnosis of GHD (9–11) in the United States and in other countries across the globe (12). Recent multi center international clinical studies regarding long-acting GH also all utilized 10mcg/dl as the GHD cut off (13, 14).

Children with a height more than 2.25SDs below the mean with no identified cause for short stature following a thorough history, physical exam, screening evaluations and GST are labelled as "idiopathic short stature" (ISS) or short stature of unknown etiology (10, 15). As children undergo more extensive evaluations, researchers proposed that the diagnosis of ISS should only be considered after detailed genetic evaluations and thorough reevaluation, including retesting with GST (16, 17).

Patients with organic brain lesions or those who have undergone cranial irradiation have been described as having GHD that evolves over time following the initial evaluation (18–22). We recently reported two patients who upon careful longitudinal monitoring and retesting of the growth hormone axis, had a diagnosis consistent with evolving IGHD without an organic brain lesion, similar to three patients previously reported by Zadik et al. (18–23). We postulate that even in patients without organic lesions careful longitudinal monitoring and retesting with GST will identify patients with findings consistent with the diagnosis of IGHD who may benefit from treatment with GH. We propose the term evolving growth hormone deficiency (EGHD) for these patients. Currently, the prevalence of this entity is unknown.

We investigated the clinical and biochemical characteristics of a cohort of 56 children followed at a single center for short stature or growth failure who had two or more GSTs due to continued suboptimal growth. We identified the characteristics of children who may be at risk for developing EGHD and evaluated their response to recombinant human GH therapy. We present adult height data in 12 of the 25 the GH treated patients with EGHD. Our findings emphasize the importance of re-evaluating children with continued evidence of inadequate growth despite a previously normal GST and who may otherwise be prematurely, and inaccurately labeled as having 'ISS'

Methods

The retrospective study was approved by the Institutional Review Board at the Icahn School of Medicine at Mount Sinai. We reviewed medical records of children who were referred to the division of Pediatric Endocrinology and Diabetes at Mount Sinai for concerns of growth (ICD-10) diagnosis code R62.50) between January 2015 and December 2020 and who underwent more than one GST as part of their evaluation. Short stature was defined as height more than 2 standard deviations (SD) below the mean for the population or for the family, using the Hermanussen and Cole definition of target height (24). And defined growth failure as patients presenting with sustained growth velocity < 0SD. Children who did not undergo more than one Growth Hormone Stimulation Test (GST), those with severe chronic illnesses (such as Inflammatory Bowel Disease, Celiac Disease, or others), or those with known genetic syndromes associated with poor growth (such as Turner syndrome, Noonan syndrome, or SHOX deficiency), as well as other identifiable causes of poor growth, were excluded from the study. None of our participants was on medications known to interfere with growth. None had received previous treatment with growth hormone. *Data Collection:* We collected clinical data including age, sex, height, and weight. BMI was calculated as weight (kg) divided by height (m) squared. Reliable height measurements were obtained by a pediatric endocrinologist using Holtain stadiometer that was calibrated weekly. Heights were plotted on the Center for Disease Control and Prevention growth chart. Height SDS and growth velocities were determined using CDC growth charts on electronic medical records. Pubertal stage (25) was obtained from the documented physical examination by the same pediatric endocrinologist and evaluated biochemically at the Endocrine Sciences laboratory. Pubertal thresholds were set at the following values: Tanner 2 breast development on examination with baseline morning LH ≥ 0.3 IU/L and baseline morning estradiol ≥ 20 pg/mL (37 pmol/L) in girls or testicular volumes ≥ 4ml with LH ≥ 0.3 IU/L and testosterone > 20ng/dL in boys (26).

Serum concentrations of IGF-1, IGFBP-3, follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estradiol, FreeT4, Thyroid stimulating hormone and cortisol, DHEA-Sulfate were obtained before each GST from Endocrine Sciences via liquid chromatography with tandem mass spectrometry(LC/MS-MS), and during follow up visits when on GH therapy. IGF-1 Z scores were calculated according to chronological age, sex and puberty.IGF-1 Z scores were calculated according to chronological age, sex and puberty; we used the LabCorp IGF-I Z-score calculator for reference. We also collected bone ages (BA) determined by pediatric radiologists as well as endocrinologists according to the standards established by Greulich and Pyle (27) based on radiographs of the left hand and wrist.

All GSTs were performed at 08.00 am following a minimum 8-hour overnight fast. Patients received a combination of two

provocative agents simultaneously the same day: 10% arginine HCL (0.5 g/kg) and oral L-dopa dose (10 mg/kg max dose of 500 mg) or IM glucagon (30 μg/kg) at time 0. Blood samples for serum GH concentrations were obtained at baseline and 30, 60, 90, 120, 150 and 180 minutes after the administration of firstagent. Serum GH was measured by the double-antibody RIA method by Endocrine Sciences Laboratory. This assay uses reagent antibodies that are polyclonal and detects 22kDA (primary GH isoform) plus other GH isoforms. This test is calibrated against International Reference Preparation (IRP) international standard (IS) 80/505 of human pituitary origin. This contains all GH isoforms. The intra- and inter-imprecision coefficient of variation are <10%.

Repeat GSTs were performed on patients who had a further decrease in growth velocity and height SDS, despite advancing puberty. Males and females were divided into GH deficient (GHD), and GH sufficient (GHS) groups based on their response to GST (Patients with peak GH of ≥ 10ng/mL were labeled as GHS and those with peak GH <10 ng/mL were labeled as the GHD cohort. Participants in the GHD cohort had Magnetic Resonance imaging (MRI) of their brain before the initiation of GH therapy evaluated by the same neuroradiologist All patients with GHD were offered GH therapy and were treated with recombinant human GH therapy (rhGH) at an initial dose ranging from 0.18 to 0.28 mg/kg/week.

Statistical analysis

We present descriptive statistics, including means and standard deviations for continuous variables, and frequencies for categorical variables. We considered a two-sided *p-value* (alpha level) threshold of ≤0.05 as statistically significant, and > 0.05 as not significant (ns). Female subjects were excluded after baseline evaluation because of the small sample size, and all subsequent analyses were performed on males only.

For continuous variables, Wilcoxon rank-sum tests (e.g., two-sample Wilcoxon tests) were used to compare treatment outcomes between GHD and GHS groups. Wilcoxon signed-rank tests (e.g., one-sample/paired-sample Wilcoxon tests) were used to compare outcomes within each GHD and GHS group to evaluate changes in growth and biochemical parameters at various time points during longitudinal follow-up. For categorical variables, chi-square tests (and Fisher's exact tests, when appropriate) were used to compare between groups, and McNemar's tests were used to compare longitudinal frequencies within groups. Data were analyzed using SPSS version 28 and SAS 9.4 (SAS Institute, Cary NC).

Results

Fifty-six patient charts were reviewed and 53 met all the inclusion criteria. Of the 53 children that were included, 42 were males (9.33 ± 2.58 years of age) and 11 were females (8.19 ± 2.76 years of age) at initial GST (Table 1). None of our patients was overweight or obese; BMI was normal at baseline as well as the end of GH treatment. Of the 42 males retested with a GST after 2.1+/-1.22 years, 25 had GHD whereas 17 were GHS. Those who had GHD, underwent MRI of the pituitary which revealed that 10 patients (33.3%) had a small pituitary; the rest were normal. There was no difference in peak GH response between those with a normal MRI versus those with small pituitary glands. Three boys were noted to be in puberty at initial GST (testicular volume >4ml and testosterone> 20 ng/dL). All the patients had puberty at a normal age. There were no outliers with respect to onset of puberty.

Table 1 shows baseline characteristics for the cohort at the initial GST. The majority of baseline characteristics between males and females were not statistically different. IGF-1 Z score on the contrary was significantly lower in the males (-0.99 ± 0.79 vs -0.22 ± 0.74, p= 0.02).

Male patients' biochemical and growth data were then compared between their initial and subsequent GSTs (Table 2), stratified by those who became GHD on repeat GST and those who remained GHS on repeat GST. There were no significant differences between the ages, mean height Z-scores and mean IGF-1 Z-scores at initial or repeat GST between the GHD and GHS groups. GHD males showed a significantly slower growth velocity (GV) of 2.98 ± 1.32 cm/yr compared to GHS males with a mean GV of 4.932 ± 1.20 cm/yr (p<0.01). There was a decrease in mean height SD in the GHD groups between GST's, decreasing from -1.68 ± 0.56SD to -1.82 ± 0.63SD, although this did not reach statistical significance, with 28% being < 2 SD at second test. In the GHS group, the height SD remained similar between GST's, from -1.88 ± 0.28SD at initial GST to -1.85 ± 0.35SD at repeat GST. For both GHD and GHS males, repeat GST was performed on average approximately 2.1 years after initial GST (Figures 1A, B).

Of the 25 GHD males, 23 were on treatment with rhGH, one patient did not start therapy during the study period. Data for GHD males were then analyzed at multiple time points after initiating rhGH treatment. 1 year after the start of treatment and at the most recent consultation visit, which was on average 2.56 ± 3.17 years after the repeat GST (Table 3). Height Z-score improved by 1.18SD during these years of GH therapy (p<0.001). GV and growth velocity standard deviation score (GVSDS) both showed significant increases at the 1-year treatment mark and at the most recent visit, 9 ± 1.54 cm/year (p<0.001) and 5.01 ± 3.13 cm/year (p<0.005), respectively. IGF-1 Z-score also showed a significant improvement (from -1.08 from the repeat GST to 1.37 at the most recent visit).

TABLE 1 Baseline patient characteristics at initial growth hormone stimulation test (GST).

Characteristics	MALES (N=42)	FEMALES (N=11)	p-value[a]
	Mean ± SD	Mean ± SD	
Age in years	9.33 ± 2.58	8.19 ± 2.76	0.29
Height Z-score	-1.76 ± 0.47	-2.01 ± 0.28	0.24
BMI Z-score	-0.48 ± 0.92	-0.59 ± 1.24	0.58
IGF-1 Z-score	-0.99 ± 0.79	-0.22 ± 0.74	**0.03**
Initial GH peak (ng/ml)	15.98 ± 5.34	16.70 ± 6.22	0.94

[a]p-value from Wilcoxon two-sample tests.
Bolded P-values indicate statistical significance.

TABLE 2 Comparison between GST 1 and GST 2 in GHD and GHS males.

Characteristics	GHD (N=25)			GHS (N=17)			GHD vs. GHS at GST1	GHD vs. GHS at Repeat GST
	GST 1	Repeat GST	p-value[a]	GST 1	Repeat GST	p-value	p-value[b]	p-value[c]
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD			
Age in years	10.07 ± 2.65	12.04 ± 2.41	**<.0001**	10.56 ± 1.81	12.44 ± 1.82	**<.0001**	0.51	0.67
Height Z-score	-1.68 ± 0.56	-1.82 ± 0.63	0.21	-1.88 ± 0.28	-1.85 ± 0.35	0.97	0.14	0.45
Testicular volume > 4ml, N (%)	5	8	0.083	3	10	**0.0082**	1.00	0.13
Testosterone in pubertal boys (N=8) (ng/dL)	76.75 ± 152.87	218.71 ± 281.21	**0.03**	7.90 ± 5.85	115.11 ± 139.50	0.06	0.43	0.47
IGF-1 Z-score	-1.05 ± 0.75	-1.08 ± 0.84	0.89	-0.90 ± 0.85	-1.09± 0.99	0.62	0.64	0.99
Growth hormone peak (ng/ml)	15.48 ± 4.92	7.59 ± 2.12	**<.0001**	16.71 ± 5.98	16.61 ± 5.39	0.93	0.54	**<.0001**

[a]p-value from Wilcoxon paired-sample test comparing GST1 to Repeat GST within groups for continuous variables, and McNemar's test for categorical variables.
[b]p-value from Wilcoxon two-sample test comparison of GHD and GHS at GST 1 for continuous variables, and chi-square test for categorical variables.
[c]p-value from Wilcoxon two-sample test comparison of GHD and GHS at Repeat GST for continuous variables, and chi-square test for categorical variables.
[d]Do not have information on growth velocity SDS or IGF-1 z-score at repeat GST.
Bolded P-values indicate statistical significance.

At the time of the repeat GST, 8 males out of 25 (32%) in the GHD group were in puberty (mean testicular volume (TV) 9.33 ± 6.0 mL). Despite entering into puberty, they continued to have poor growth velocity of 3.34 ± 1.46 cm/year ($p<0.05$) and Height Z score -1.46 ± 0.75 SD (Supplementary Table 1). On GH treatment, the Height Z score in this cohort had dramatically improved to 0.48 ± 0.27 SDs ($p= 0.0625$) within one year of start of treatment and 0.04 ± 1.59 SDs ($p<0.05$) at the most recent visit (2.20 ± 1.80) SDS. Growth velocity improved to 8.84 ± 2.43 cm/year at one year of treatment and 4.33 ± 3.13 cm/year at most recent visit (Supplementary Table 1). The difference between bone age and chronological age changed from -1.00 ± 1.04 years to -1.96 ± 1.27 years at the most recent visit ($p< 0.05$). Further, the IGF-1 Z score had also improved from -1.27 ± 1.19 to 0.92 ± 2.18 SDS ($p=0.016$). Seventeen males (68%) had not yet entered puberty (mean TV 3.00 ± 1.51) with an average repeat GST of 7.91 ± 1.92 ng/mL. There were no significant differences between mean GST GH peak, height SDS, GV, GVSD or IGF-1 Z-score at repeat GST between pubertal and non-pubertal males (Supplementary Table 1). Although not statistically significant, these parameters improved with GH therapy in both the pubertal and non-pubertal groups.

Twelve GHD males treated with rhGH reached adult height (AH).They were treated on average for 4.64 ± 1.36 years. They achieved an average adult height Z-score of 0.08 ± 0.69, an average

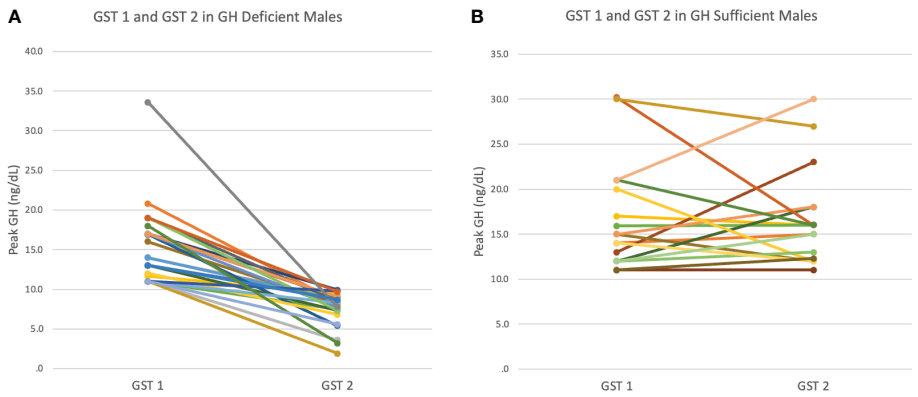

FIGURE 1
(A) Peak GH response at first (GST1) and second (GST2) growth hormone stimulation test in GH Deficient (GHD) males. **(B)** Peak GH response at first (GST1) and second(GST2) growth hormone stimulation test in GH Sufficient (GHS) males.

TABLE 3 Patient characteristics for boys with GHD at different time points (n=24).

Characteristic	Repeat GST	1 year after GH	p-value[a]	Most recent visit	p-value[b]
	Mean ± SD	Mean ± SD		Mean ± SD	
Height Z-score	-1.82 ± 0.63	-1.37 ± 0.58	**<.0001**	-0.64 ± 1.08	**<.0001**
Height SD gain	n/a	0.55 ± 0.22	n/a	1.21 ± 0.99	0.06
Growth velocity (cm/year)	2.98 ± 1.32	9.00 ± 1.54	**<.0001**	5.01± 3.13	**0.005**
Growth velocity SDS	-1.19 ± 1.51	N/A[c]	N/A	0.82 ± 1.66	**0.002**
IGF-1 Z-score	-1.08 ± 0.84	N/A	N/A	1.36 ± 1.67	**<.0001**

*Height values in this table calculated as change from height right before GH stim.
[a]p-value from Wilcoxon paired-sample test comparing repeat GST to 1 year after GH.
[b]p-value from Wilcoxon paired-sample test comparing most recent visit to repeat GST.
[c]Do not have information on growth velocity SDS or IGF-1 z-score at 1 year after GH.
p-value[a]: Statistical significance between Repeat GST and 1 year after GH therapy.
p-value[b]: Statistical significance between Repeat GST and most recent visit.
Time since most recent visit is 2.56 ± 3.17 years.
Bolded P-values indicate statistical significance.

adult height of 176.53 ± 5.33 cm (Table 4). Both, adult height Z-score and adult height were statistically significant when comparing adult height to height at repeat GST(p=0.0005). With GH treatment, the average gain in height SD was 1.85 ± 0.55SD (p<0.001). The adult height was on average, 2.03 ± 4.4 cm above the mid-parental target height (MPTH).

There were too few females in our cohort, and hence the analyses were only limited to male patients. This is consistent with gender disparities, reported previously in idiopathic GHD (28).

Discussion

Most cases of GHD in pediatric patients are idiopathic, isolated and acquired. All acquired conditions commence with a period during which the diagnosis may not be apparent. However, this may evolve over time (29). We define EGHD as a condition characterized by the progressive decline or insufficient production of growth hormone (GH), especially during the period of pubertal development. It is diagnosed when individuals who were initially deemed GH-sufficient continue to grow inadequately and display signs of inadequate GH secretion, such as decreased growth velocity for age and sex, reduced height SD despite being in puberty, and/or delayed skeletal maturity and are deficient upon GH retesting with GST. We hypothesize that EGHD may be due to evolving hypopituitarism, as the pituitary may be unable to support the compensatory response to normal growth and puberty. EGHD is distinct from ISS and constitutional growth delay due to its unique clinical presentation and responsiveness to GH therapy.

TABLE 4 Adult height data in GHD males when bone age ≥ 16 years with growth hormone therapy for an average 4.64 ± 1.36 years (n = 12).

Characteristic (by variable name)	N = 12
	Mean ± SD
Adult Height	176.53 ± 5.33
Adult Height SD	0.08 ± 0.69
Mid parental height	173.77± 3.08
Height at Repeat GHST	139.72 ± 13.88
Height SD at Repeat GHST	-1.75 ± 0.73
Height SD before GH treatment	-1.88 ± 0.64
Adult height – Height at Repeat GHST (Delta Height in cm)	36.82 ± 11.35
Adult height - mid parental height	2.10 ± 4.20
Adult height SD – Height SD at GH start	1.86 ± 0.58

p-value[c]: comparing adult height to repeat GST or mid parental target height.

We document here evidence for evolution of GHD in a group of 42 adolescent males who tested sufficient on initial GH stimulation test (GST) but 59% tested deficient upon reevaluation and had an excellent response to GH therapy. Therefore, we considered this an evolving process. Importantly, 32% of these GHD boys were already pubertal when we reevaluated. Clinically, while most characteristics remained similar between boys who tested GHD vs GHS, those with GHD did have a decrease in height Z-score and GV unlike the GHS boys at the repeat GST. Deficient males were treated with GH therapy and demonstrated improvement in height Z-score and growth velocity. All our patients tolerated the treatment well without any adverse effects.

GHD after repeat GST was first reported in patients with organic brain lesions and those who underwent cranial irradiation (18–22, 30). The same phenomenon in the absence of an organic brain lesion or cranial irradiation was first described by Zadik et al. in 1997 (9). This case series highlighted the importance of follow-up of children with unidentified causes of short stature and retesting with GST, especially since they may have been termed as 'Idiopathic short stature' otherwise. Our team recently reported two male patients (also currently included in this study) with poor growth and pubertal progression who initially had normal GST but were later diagnosed with EGHD and benefitted from GH therapy (28). GSTs are useful tools in diagnosing GHD, especially in those without obvious causes of GHD such as intracranial tumors or irradiation (9). We acknowledge that several studies of various GH stimulation test protocols in different parts of the world have suggested different GH peak cut offs ranging from 5 to 10 ng/dL

(31). But for all practical purposes the current agreed GH cut off in the United States remains to be 10 ng/dL (32). The current published literature on long-acting GH research is also based on the GHD cut off of 10 ng/dl (13, 14). In our patient cohort 36% had a peak ≤ 7 ng/dL on repeat GST; had we waited extended periods, perhaps they may have had lower GH peak levels. Further, to make a diagnosis of GHD in our practice, we also rely on various parameters such as height Z-score, growth velocity, stage of puberty, MPTH, and biochemical markers (such as IGF-1) in addition to the GST. Hence, long-term follow-up and repeat GST along with careful consideration of other biochemical parameters are key to diagnosing patients with EGHD. Treatment may benefit these patients in reaching their expected height potential.

We considered if our patients' presentation was a result of constitutional delay in growth and puberty (CDGP). However, unlike patients with CDGP, our patients had poor growth velocities in addition to short stature for age or family, while puberty was progressing (33). This is further supported by a study from Binder et al. who compared growth velocities in patients with CDGP and organic GHD from National Cooperative Growth Study (NCGS) registry (n=164) (34). They found that patients with organic GHD had significantly lower height velocity of 3.5 ± 3.2 cm/year compared to height velocity of 5.2 ± 5.4 cm/year in those with CDGP (p <.0001) at 14.1 ± 0.4 age. Our prepubertal males with EGHD had a slower growth velocity of 3.34 ± 1.46 cm/yr., comparable to patients with true GHD in the NCGS cohort. Further, growth velocity is typically restored when in puberty for patients with CDGP, but despite 32% of our males being in puberty, their growth velocity was 2.69 ± 0.99 cm/yr. (refer Additional Table 1) (33). In a French registry, males treated with GH at a mean chronological age of 13.2 ± 2 years, had a mean height gain of 1.1 ± 0.9 SDS resulting in an average AH of -1.6 SD (boys 165 ± 6 cm) (35). The AH Z-score was 0.4 SD lower than MPTH Z-score and there was a significant concern that the majority of the patients had a constitutional delay of growth and puberty. Hence, although patients with CDGP may receive GH for various concerns, their response is not as robust as those with true GHD, such as our cohort (AH gain of 1.9 SDs) (18, 36).

After one year of treating our patients with GH, the average growth velocity was comparable to the NCGS database, reported as 10.0 ± 1.03 cm/year in their IGHD population at the same age as our population (37). The KIGS database (Pfizer International Growth Databases) reported that children grew at 8.4 ± 2.08 cm/year with GH therapy, similar to our cohort. However, their patients were significantly younger(7.78 ± 2.93 years) compared to our patients (12.04 ± 2.41years) (30). In contrast, patients with ISS after one year of GH treatment were reported to grow at 7.8 ± 2.6 cm/year, slower than our cohort of GHD boys (38, 39). The height Z-score gained) in those with GHD compared to ISS were much higher(0.62 ± 0.33 vs 0.40 ± 0.27, p = 0.03), similar to our patients (0.55 ± 0.22 SDS). Pfäffle et al. compared studies between GHD and ISS patients on GH therapy of which one study reported a height gain of 1.3 SDS over an average of 4.6 years in patients with IGHD while another study reported a gain of only 0.5 SD in patients with ISS over an average of 4.4 years (24). Both the KIGS and NCGS GHD patient cohorts reported mean adult height SDS of -3.1 to 0.2 SDS (40) and -0.7 ± 1.3 (41) respectively, lower than our data after 8.1 years and 4.6 years respectively. Furthermore, Loche et al. studied 15 prepubertal non-GHD children with short stature who were treated with GH and reported their adult heights were not more than their target heights despite having a high mean growth velocity during the first year of therapy (42). A Cochrane review suggested that adult height in patients with ISS is usually lower than their MPTH when compared to individuals with normal stature, hence indicating that it is typically the patients with GHD who are able to reach their genetic potential with GH therapy (43). Our cohort's response to GH therapy was very similar to multiple reports of patients with GHD, confirming the diagnosis of EGHD All 12 had normal IGF-1 levels measured 3 months or more after therapy and hence did not have repeat GHST. This finding is consistent with previous reports (44).

Our study, additionally supported by adult height data, is a proof-of-concept for the diagnosis of EGHD. We are not aware of previous such reports reported in the literature. In our study we were able to longitudinally follow up almost half of our patients with EGHD to adult height, showing successful therapy with GH without any adverse events. We provide information regarding the characteristics of individuals with EGHD which could be helpful to clinicians in diagnosing and treating this condition.

One of the major limitations of this study is that it is retrospective. In our practice, we do not routinely prime our prepubertal children during GST given the lack of consensus for additional benefit (10, 12, 45, 46). However, eight of our males 32% indeed self-primed as they entered puberty with testosterone reaching pubertal levels and yet tested GHD. We further acknowledge the absence of a control group, as it was not possible to compare outcomes of the treatment with a group of patients who did not receive treatment as they were lost to follow up. We recognize the limitations in reproducibility associated with the Growth Hormone Stimulation Test (GST) and, therefore, integrate the results of GST into a comprehensive clinical and biochemical assessment that is in alignment with the diagnostic criteria for growth hormone deficiency (GHD). We are aware of the lack of consensus regarding the diagnosis of GHD. Several recent publications including multicenter and national studies have used 10 ng/dL as the cut off on GST for GHD diagnosis, as we've used in this study (9–12). Lastly, we didn't explore genetic factors contributing to the development of EGHD, as it is not yet routine to pursue genetic evaluation in individuals with idiopathic, isolated growth hormone deficiency. A recent article by Murray et al. suggested that some causes for short stature in patients with ISS could be explained via genetic testing, which we intend to explore in future studies (47).

In conclusion, we describe a new entity of EGHD among patients evaluated for short stature. We also provide adult height data in the half who have reached adult height that are compatible with results reported for those with IGHD, supporting the diagnosis. Therefore, we strongly recommend that EGHD be considered as part of the differential diagnosis for short stature. Longitudinal careful monitoring and retesting, when necessary, will identify those with EGHD who will benefit from rhGH therapy and will avoid the premature classification of some as ISS.

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 humans were approved by Icahn School of Medicine at Mount Sinai, Grant Office ID: 19-2771. 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

SC: Writing – review & editing, Writing – original draft. SS: Writing – original draft, Formal analysis, Data curation, Conceptualization. JS: Writing – review & editing, Data curation. AK: Writing – review & editing, Formal analysis. CM: Writing – review & editing, Visualization, Data curation. MS: Writing – review & editing. RR: Writing – review & editing, Writing – original draft.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1398171/full#supplementary-material

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Recommendations for recognizing, risk stratifying, treating, and managing children and adolescents with hypoglycemia

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There has been continuous progress in diabetes management over the last few decades, not least due to the widespread dissemination of continuous glucose monitoring (CGM) and automated insulin delivery systems. These technological advances have radically changed the daily lives of people living with diabetes, improving the quality of life of both children and their families. Despite this, hypoglycemia remains the primary side-effect of insulin therapy. Based on a systematic review of the available scientific evidence, this paper aims to provide evidence-based recommendations for recognizing, risk stratifying, treating, and managing patients with hypoglycemia. The objective of these recommendations is to unify the behavior of pediatric diabetologists with respect to the timely recognition and prevention of hypoglycemic episodes and the correct treatment of hypoglycemia, especially in patients using CGM or advanced hybrid closed-loop systems. All authors have long experience in the specialty and are members of the Italian Society of Pediatric Endocrinology and Diabetology. The goal of

treating hypoglycemia is to raise blood glucose above 70 mg/dL (3.9 mmol/L) and to prevent further decreases. Oral glucose at a dose of 0.3 g/kg (0.1 g/kg for children using "smart pumps" or hybrid closed loop systems in automated mode) is the preferred treatment for the conscious individual with blood glucose <70 mg/dL (3.9 mmol/L), although any form of carbohydrate (e.g., sucrose, which consists of glucose and fructose, or honey, sugary soft drinks, or fruit juice) containing glucose may be used. Using automatic insulin delivery systems, the oral glucose dose can be decreased to 0.1 g/kg. Practical flow charts are included to aid clinical decision-making. Although representing the official position of the Italian Society of Pediatric Endocrinology and Diabetology (ISPED), these guidelines are applicable to the global audience and are especially pertinent in the era of CGM and other advanced technologies.

KEYWORDS

adolescents, automated insulin delivery, children, hypoglycemia, glucagon, oral glucose, type 1 diabetes

1 Introduction

There has been continuous progress in diabetes management over the last few decades. The widespread dissemination and adoption of continuous glucose monitoring (CGM) and automated insulin delivery (AID) systems have completely changed the daily lives of patients with diabetes, improving the quality of life of both children and their families. Despite this, hypoglycemia remains the primary side-effect of insulin therapy. The American Diabetes Association defines hypoglycemia as "all episodes of an abnormally low plasma glucose concentration that expose the individual to potential harm", highlighting both the physical and psychological/psychosocial dangers. In addition to the adverse effects (i.e., need to interrupt daily activities, unpleasant symptoms, social embarrassment, loss of consciousness, seizures), hypoglycemia may lead to the development of fear of hypoglycemia (FoH). Hypoglycemia and FoH represent the main obstacles to achieving optimal metabolic control. Therefore, the primary objectives of hypoglycemia management for the diabetes care team are timely recognition of hypoglycemic episodes, their prevention, and administering the correct therapy.

The objective of this work is to unify the behavior of all Italian pediatric diabetologists with respect to the timely recognition and prevention of hypoglycemic episodes and the correct treatment of hypoglycemia, also focusing on the psychophysical wellbeing of patients and their families. To achieve this, the Study Group on Diabetes of the Italian Society of Pediatric Endocrinology and Diabetology (ISPED) decided to draw up this set of recommendations on "Hypoglycemia in Children and Adolescents with Diabetes". The evidence presented within these recommendations is based on a systematic review of the available scientific evidence and a Delphi consensus methodology that involved all participants listed as authors and in the Appendix. All relevant papers published in the last 5 years (January 1st, 2019 to December 31st, 2023) were carefully evaluated by all authors and were used to inform each section of these recommendations.

In 2022, the International Society for Pediatric and Adolescent Diabetes ISPAD released their Clinical Practice Consensus Guidelines on the assessment and management of hypoglycemia in children and adolescents with diabetes (1). Although these recommendations align with ISPAD, we also provide additional information on rapidly evolving areas of clinical interest including fear of hypoglycemia (FoH), nasal glucagon use, and educational support to fully address the needs of the local (Italian) context.

The ISPED Advisory Council approved this document, and it therefore represents its official position.

2 Defining hypoglycemia and its incidence

2.1 Definition

The American Diabetes Association defines hypoglycemia in diabetes non-numerically as "all episodes of an abnormally low plasma glucose concentration that expose the individual to potential harm" (2). Nevertheless, it is essential to identify a level of hypoglycemia that should be avoided due to its immediate and long-term impact on the individual (1). Numerical definitions (Table 1) are based on glucose values detected by self-monitoring blood glucose (SMBG), CGM, or laboratory measurement of plasma (2). Hypoglycemia is *symptomatic* when the child, adolescent, or parent notices the presence of one or more symptoms and verifies that the blood glucose is <70 mg/dL (3.9 mmol/L).

TABLE 1 Definition of hypoglycemia and clinical targets for CGM data.

Definition	Clinical hypoglycemia alert	Clinically important hypoglycemia	Severe hypoglycemia
Threshold	<3.9 mmol/L or <70 mg/dL	<3.0 mmol/L or <54 mg/dL	No specific threshold but linked to symptoms
CGM target for hypoglycemia	<4% or <1 hour/day	<1% or <15 min/day	

A hypoglycemic episode can be defined as:

1) <70 mg/dL (3.9 mmol/L): clinical hypoglycemia alert, used as the threshold value for identifying and treating hypoglycemia.
2) <54 mg/dL (3.0 mmol/L): clinically important or serious hypoglycemia. Neurogenic symptoms and cognitive dysfunction occur below this level, together with an increased risk of severe hypoglycemia (3, 4).
3) Severe hypoglycemia: event characterized by altered mental and/or physical status (including coma and seizures) that requires assistance for resolution.

Note that as young children require assistance to correct even mild hypoglycemia, the event requires the caregiver and physician to evaluate whether the child has hypoglycemia-induced cognitive dysfunction.

2.2 Incidence

Mild hypoglycemia is common and asymptomatic events are likely to be underreported, making the exact incidence of hypoglycemia difficult to establish. However, symptomatic hypoglycemia is estimated to occur on average twice a week in >80% of people with diabetes, with countless episodes in a lifetime (5). There has been a significant reduction in the incidence rates of hypoglycemia in international registries over the last two decades (6–8).

3 Signs and symptoms

The signs and symptoms of hypoglycemia in people living with diabetes (PWD) are caused by adrenergic activation when whole blood glucose falls to 65-70 mg/dL (3.6-3.9 mmol/L) and neuroglycopenia due to glucose deprivation in the brain (9) (Table 2).

The plasma glucose threshold for activation of counter-regulatory hormone secretion is thought to be higher than for initiation of autonomic warning symptoms (~70 mg/dL vs. ~60 mg/dL, respectively) (4). However, a recent systematic review (11) challenged this assumption, with release of counter-regulatory hormones in young adults with type 1 diabetes (T1D) occurring at a median plasma glucose level of 50-61 mg/dL and generation of both autonomic and neuroglycopenic hypoglycemic symptoms starting at a similar glucose level of around 54 mg/dL. These values are lower than those of non-diabetic subjects.

TABLE 2 Hypoglycemia signs and symptoms (3) (adapted from Abraham MB, ref. 10).

Autonomic	Shakiness, sweatiness, trembling palpitations, pallor
Neuroglycopenic	Poor concentration Blurred or double vision, disturbed color vision Difficulty hearing Slurred speech Poor judgment and confusion, problems with short-term memory Dizziness and unsteady gait Loss of consciousness, seizure, death
Behavioral	Irritability, erratic behavior, agitation, nightmares, inconsolable crying
Non-specific	Hunger, headache, nausea, tiredness

Within the first year of T1D, glucagon responses to hypoglycemia are blunted but epinephrine responses are not; defective and absent glucagon responses to hypoglycemia have been observed in PWD with significant residual endogenous β-cell function (12, 13). In children with T1D, the coalescence of autonomic and neuroglycopenic symptoms may indicate that both responses are generated at similar glycemic thresholds (14). Neuroglycopenic symptoms were reported more commonly in PWD who reported partial awareness of hypoglycemia than those who reported normal hypoglycemia awareness; by contrast, autonomic symptoms were reported less frequently by PWD who had hypoglycemia unawareness (15). Young children typically do not have hypoglycemia awareness or do not have the vocabulary to describe how they are feeling, so it is important to be vigilant for behavioral changes or signs (such as pallor) associated with hypoglycemia (14). The glycemic threshold for hypoglycemia symptoms may occur at a different glucose level in children for different reasons (Table 3).

3.1 Severe hypoglycemia

In pediatric PWD, severe hypoglycemia is characterized by convulsions, coma, or other neurological symptoms of neuroglycopenia, and it requires therapy with glucagon or IV glucose (17). Risk factors for severe hypoglycemia are age, diabetes duration, glycemic control, type of treatment, unawareness, nighttime episodes, exercise, and previous episodes (10). Other possible risk factors include risky behaviors (alcohol, recreational substances, lack of preparation for sports, infrequent blood glucose monitoring, etc.), as described further below. While

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TABLE 3 Variables that influence threshold for onset of symptoms.

Age	Symptoms of hypoglycemia and physiological counter-regulatory hormone release occur at a higher glucose level in children than in adults (16).
Duration of diabetes	The symptoms of hypoglycemia lessen with increasing duration of diabetes. There is a progressive loss of glucagon response to insulin-induced hypoglycemia over the 12 months after diabetes onset and it is lost in most people with T1D by 5 years (17, 18). Thus, half of adult patients with long-term diabetes have experienced unawareness of hypoglycemia, leading to severe episodes (19), while no data are available for children and adolescents.
Chronic hyperglycemia	Chronic hyperglycemia and poor glycemic control can result in an adaptive shift of the threshold of onset for hypoglycemic symptoms to a higher glucose level (20) than in persons without diabetes (21), up to normoglycemic levels (16).
Recent hypoglycemia	Glycemic thresholds for symptoms of hypoglycemia shift to lower plasma glucose concentrations after recent antecedent hypoglycemia (22). Hypoglycemia begets hypoglycemia, and recurrent episodes of mild hypoglycemia contribute to the development of defective counter-regulatory hormone responses to subsequent reductions in blood glucose levels (23).

younger children are thought to be at higher risk of severe hypoglycemia (24), some studies have not confirmed this association (25–27). Severe hypoglycemia is probably more frequent in adolescents because of the longer duration of disease and higher insulin requirements. Low HbA1c is no longer considered a risk factor for severe hypoglycemia in young PWD since the advent of AID, but a low HbA1c value must always be carefully considered in the clinical context; on the contrary, severe hypoglycemia is followed by a progressive and lasting increase in HbA1c in children and adolescents with T1D (26). People with T1D treated with five or more daily insulin injections were shown to be at reduced risk of severe hypoglycemia compared with subjects on fewer daily injections (24). People with T1D on insulin pumps are at reduced risk of severe hypoglycemia (16), and no episodes of severe hypoglycemia were observed in most hybrid closed-loop (HCL) or advanced hybrid closed-loop (AHCL) trials (18, 19, 28).

4 Risk factors

There are several non-modifiable risk factors for hypoglycemia, including younger age, long duration of diabetes, and comorbidities. Several risk factors are, however, modifiable, for example the type of insulin treatment and insulin doses, physical activity, dietary habits, drug use or substance abuse, and others.

4.1 Alcohol and hypoglycemia

Alcohol use is common in adolescents with T1D. Since alcohol intake is more frequent during evening hours and plasma glucose mainly decreases 8-12 hours after ethanol administration (20), the risk of hypoglycemia is higher during subsequent sleep hours. Binge drinking represents one end of the spectrum of alcohol consumption, and it is more frequent in males (21). In the DPV registry of youths and young adults with T1D, alcohol use was associated with worse glycemic control, more severe hypoglycemia, and increased rates of diabetic ketoacidosis (DKA) (22).

Even though the combination of alcohol intake and fasting is assumed to induce hypoglycemia, there are few real-time studies on the topic. Garcia et al. (23) reported that moderate alcohol consumption (0.7 g of alcohol per kg of body weight, given as beer) with a mixed meal does not seem to increase the risk of postprandial hypoglycemia over at least six hours post-ingestion. A systematic review applying GRADE criteria by Tetzschner et al. (29) examined studies of alcohol-induced hypoglycemia in PWD and found that most recommendations on hypoglycemia prevention strategies were based on best clinical practice rather than on objective evidence. Overall, the first advice for subjects with T1D is to take precautions when consuming alcohol: the best prevention for alcohol-induced hypoglycemia is, in fact, an awareness of its hypoglycemic effects, especially when it is drunk without simultaneous ingestion of carbohydrates.

4.2 Exercise

Exercise can increase the risk of hypoglycemia via several mechanisms: increased glucose consumption, depletion of glycogen stores, increased insulin sensitivity, and exercise-induced counterregulatory hormone deficits (30). The increased risk of hypoglycemia and FoH are a barrier to exercise for PWD (31). The risk of hypoglycemia is greater with aerobic or endurance exercises than with anaerobic or high-intensity exercises (32). The intensity, duration, and type of physical activity, timing to and site of insulin infusion/injection, carbohydrate intake, glucose profile pre-exercise, type of insulin, insulin treatment (MDI/CSII/AID), hydration status, level of training, and age influence the personal risk of hypoglycemia during exercise (33, 34). The risk of hypoglycemia increases with moderate-intensity exercise, immediately after activity, and 7 to 12 hours after exercise (35).

The risk of hypoglycemia is further increased by the reduced counter-regulatory response induced by exercise itself and during sleep (36). Optimizing the glucose profile is fundamental: the appropriate reduction in insulin (basal and bolus insulins) before and after exercise, adequate intake of carbohydrate (before and after exercise), and monitoring of glucose profiles, recognizing that all glucose sensors are less accurate during exercise and in the hypoglycemic range (35).

Another strategy to minimize exposure to hypoglycemia after exercise is to plan exercise sessions at high intensity: counter-regulatory hormones can, in fact, increase endogenous glucose production and maintain glycemia at a higher range than sessions of moderate-to-intense exercise alone (37).

4.3 Nocturnal hypoglycemia

Nocturnal hypoglycemia has always been scary for PWD and their parents, significantly affecting their sleep quality and, consequently, quality of life (38). New long-acting insulins have significantly reduced the risk of nocturnal hypoglycemia, especially when compared to NPH insulins. More specifically, degludec and U-300 glargine best prevent nocturnal hypoglycemia in children

(39). Furthermore, advances in CGM technology and alarm systems have significantly reduced the risk of hypoglycemia at night (40). The use of HCL with low-glucose suspend (LGS) and the possibility of establishing a specific sleep-hours algorithm have significantly diminished the risk of hypoglycemia (41).

4.4 Others

In case of unexplained and repeated hypoglycemic episodes (42), celiac disease (43), Addison's disease (44), hypothyroidism (45), and factitious hypoglycemia (including Munchausen-by-proxy) should be considered. However, the evidence that subclinical hypothyroidism is a cause of repeated hypoglycemia is weak.

5 Treatment of severe hypoglycemia

The primary goal of treatment is to raise blood glucose above 70 mg/dl and to prevent further decreases in blood glucose levels (42). After a severe hypoglycemia episode, it is essential to discuss the reasons why the episode occurred with the subject and caregivers. In addition, close follow-up and regular glucose monitoring are necessary in the days and weeks after the episode.

5.1 In-hospital treatment

In a hospital setting, intravenous glucose must be immediately administered to maximally limit exposure to hypoglycemia. The recommended dose of dextrose (glucose) is 0.2 g/kg; this dose can reverse hypoglycemia without the risk of unintentional osmotic diuresis (2). It is essential to pay attention to the concentration of glucose solution and the infusion rate. Highly concentrated glucose solutions (dextrose 50%) or infusion rates >5 mg/kg/min should be avoided due to the risk of excessive rate of osmotic change and, consequently, the risk of hyperosmolar cerebral injury (46). Moreover, highly concentrated glucose solutions can cause peripheral vein sclerosis, so administering glucose solutions at concentrations greater than 25% dextrose is not recommended. 10% dextrose has been shown to be effective and safe for treating hypoglycemia in a randomized controlled trial (47). The recommended dose of dextrose (0.2 g/kg) equals 2 mL/kg 10% dextrose solution. The maximum dose is 0.5 g/kg of body weight, corresponding to 5 mL/kg. In cases of recurrent hypoglycemia with the inability to take an adequate amount of carbohydrates orally, it is possible to prolong the intravenous infusion of 10% dextrose with a glucose infusion rate of 2-5 mg/kg/min (1.2-3.0 mL/kg/h).

5.2 Treatment at home and school

At home and in school, severe hypoglycemia should be immediately treated by administering glucagon. Glucagon can rapidly reverse severe hypoglycemia, except in situations of liver glycogen depletion after prolonged fasting, where intravenous administration of glucose solutions is more effective (48).

Children and adolescents with T1D of all ages with severe hypoglycemia can be treated with intramuscular (IM) or subcutaneous (SC) injection of recombinant crystalline glucagon available as a lyophilized (freeze-dried) powder. This formulation needs to be reconstituted to a concentration of 1 mg/mL with sterile water in a series of multiple steps immediately prior to injection. Two commercial glucagon rescue kits are currently available in Italy: GlucaGen® HypoKit 1 mg (Novo Nordisk®A/S, Bagsvaerd, Denmark) and Glucagon Emergency Rescue Kit (Baqsimi, formerly Eli Lilly and Company, Indianapolis IN, USA, now Amphastar Pharmaceuticals, Inc., Rancho Cucamonga, CA, USA). The recommended dose of glucagon depends on body weight: adults and children >25 kg should receive 1 mg, whereas children should be treated with 0.5 mg. Nasal glucagon can be used from 4 years of age and above.

In recent years, intranasal (IN) glucagon has been increasingly used due to its easier administration. The IN formulation (Baqsimi™) is composed of glucagon as a powder with beta-cyclodextrin plus dodecylphosphocholine as the promoter for nasal absorption. Clinical trials have demonstrated that administration of IN glucagon is safe and effective for raising blood glucose levels during moderate hypoglycemia episodes under controlled conditions in adults (49) and children (50) with T1D, without being affected by the common cold and concomitant administration of nasal decongestant. Moreover, simulation studies have shown that the administration of nasal glucagon is faster and easier than injectable glucagon (51). The recommended dose of IN glucagon is 3 mg, equal to one puff. A recent meta-analysis demonstrated that intranasal glucagon and subcutaneous (SC)/intramuscular glucagon were equally effective for treating hypoglycemia (52). Moreover, additional real-life evidence has demonstrated the efficacy of IN glucagon in a large cohort of Italian children and adolescents (53). Common side-effects of IM and SC recombinant crystalline glucagon are nausea and vomiting and, in addition to these known side-effects, nasal glucagon may cause headache, upper airway discomfort, or nasal congestion (53).

5.3 Treatment of mild-to-moderate hypoglycemia

Mild-to-moderate hypoglycemia should be treated with rapidly absorbed carbohydrates. Subjects should re-test and re-ingest carbohydrates every 15 minutes until they recover from hypoglycemia (Figure 1).

The recommended dose of carbohydrate for children and adolescents is 0.3 g/kg oral glucose, which has been shown to be effective in increasing glucose levels by around 36 mg/dL within 15 minutes of ingestion in children (54). This approach was also found to be effective in children on insulin pumps (55).

Glucose-containing products (e.g., Glucosprint, Fastup, etc) are more effectively and quickly increase glucose levels than sucrose and fructose-containing products (56, 57). Glucose-containing tablets or drinks are currently not reimbursed by the National Health System

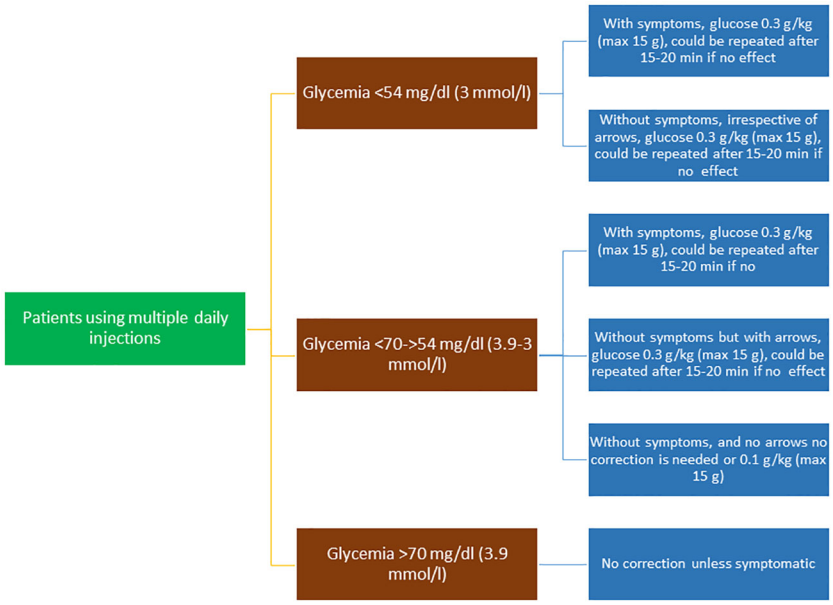

FIGURE 1
Flow chart of the treatment of hypoglycemic episodes in patients using multiple daily injections.

in Italy, and they are more expensive than non-glucose-containing products. For these reasons, dietary sucrose and fructose-containing products (e.g., candy, sugar cubes, juice) are more frequently recommended in daily clinical practice. Both sucrose and fructose are required in greater amounts to provide the same increase in blood glucose compared with oral glucose (e.g., 20 g in the form of glucose tablets corresponds to 40 g of juice). Moreover, the rise in glycemia after sucrose ingestion is around half as fast as after glucose ingestion and could be even slower with fructose (56).

Parents of toddlers sometimes use honey and milk, but this approach should be discouraged: honey has a fructose content of around 70%, and the total sugar content is highly variable. Milk contains approximately 5 g of carbohydrate in 100 mL, and it causes a minimal rise in glycemia (around 4-5 mg/dL) (58). Complex carbohydrates and foods containing fats (e.g., chocolate) should also be avoided due to delayed intestinal absorption and the slow rise in glucose obtained.

5.4 Mini-dose glucagon

In cases of nausea, vomiting, or food refusal, small doses of subcutaneous glucagon can be administered to restore plasma glucose to normal. Using a standard U-100 insulin syringe, two units (20 µg) for children ≤ 2 years and 1 unit/year for children aged 3-15 years to a maximum dose of 150 µg or 15 units can be administered subcutaneously. If the blood glucose does not increase within 30 minutes, the initial dosage can be repeated. These dosage and treatment protocols are safe and effective in children, including toddlers, and increase glucose by 60-90 mg/dL within 30 minutes of administration (59).

5.5 Hypoglycemia treatment at school

School personnel should receive an appropriate diabetes education program to identify hypoglycemia signs and symptoms. In the "Hypoglycemia" section of the Diabetes Management Plan, it is essential to define individual signs and glycemic values that define intervention and glucagon use (60). Blood glucose meters should be available in school as a "first aid hypoglycemia management pack" containing glucose, glucose tablets, fast-acting sugar sources, and extra snacks, which should be available in the classroom or the bag of the child or adolescent with T1D.

5.6 Treatment using AID systems

Although AID systems have been shown to reduce episodes of hypoglycemia (19, 20, 28), they do not completely avoid them. Automated systems can reduce insulin delivery to zero for a period such that, during a hypoglycemia episode, there is usually less active insulin than with traditional insulin pump therapy. However, using these advanced systems, an excess of insulin and consequent hypoglycemia due to human mistakes (for example, a wrong bolus dose) or an unplanned intense physical activity can occur.

Although the traditional oral treatment of mild-to-moderate hypoglycemia in a conscious child involves a correction of 0.3 g/kg of glucose in a PWD using an insulin pump integrated with advanced algorithms, this correction is not always required. It is only necessary in a hypoglycemia alert in symptomatic subjects or within two to three hours of a bolus with excess active insulin. In real life, the current approach is correction with simple sugar/glucose at a dosage of 0.1 g/kg, resulting in 4-8 g of total

carbohydrates, but waiting at least 15 minutes before treating to avoid glucose value oscillations. This practical advice arises from two considerations: these systems, in particular AID systems, continue to intervene in the administration of insulin, and subjects are significantly less insulinized than with conventional therapy; therefore, given that the system is reactive to blood sugar, if a sudden increase is detected (hypercorrection), an automatic corrective bolus is delivered that can help propagate new hypoglycemia (61, 62). Moreover, AID systems and sensors in general significantly reduce both severe and mild-to-moderate hypoglycemia episodes (61, 62), especially in the context of a structured educational program (62).

A summary of recommendations for hypoglycemia treatment in children and adolescents using AID systems is shown in Table 4 and Figure 2.

Since alterations in glucose homeostasis might reduce gray and white matter volume and alter brain metabolism, it is important to note that hyperglycemia more than hypoglycemia can damage the brain. This is particularly interesting in relation to the therapeutic habit of preferring higher blood sugar levels in children with T1D rather than risk unwanted hypoglycemia. In this respect, using an AID system, especially in young children with T1D, can help to prevent both hypo- and hyperglycemia (63).

6 Prevention

Hypoglycemia is preventable because it is frequently predictable (1). Approaches to preventing hypoglycemia include glucose monitoring, patient education, meal planning, insulin therapy adjustment, glucose sensors, and AHCL pumps. To prevent hypoglycemia, diabetes education is essential. All children and adolescents and their caregivers should be educated on the risk factors for hypoglycemia to alert them to the times and situations where increased glucose monitoring is required and when treatment regimens need to be adjusted (3). For documented hypoglycemia without symptoms or impaired awareness of hypoglycemia, parents should contact their diabetes care team to review the care plan. To prevent hypoglycemia, it is extremely important to set AHCL systems to "activity" or "exercise" mode or set a temporary basal rate. These settings must be reevaluated periodically.

To prevent hypoglycemia, it is essential to encourage regular meal consumption and teach PWD how to count the carbohydrates contained in foods to administer the correct insulin dose. In addition to carbohydrates, fat and alcohol intake can influence glycemic trends.

TABLE 4 Recommendations for hypoglycemia treatment in patients using HCL/AHCL systems.

WHEN	>15 minutes at <70 mg/dL, hypoglycemia alert event
START	Treatment with 4-8 g carbs (0.1-0.15 g/kg)
EXCEPTIONS (more carbs)	- Hypoglycemia with exercise - When the PWD suspects a significant overestimation of carbs/meal bolus
WAIT	15 minutes before re-treating hypoglycemia to avoid oscillating glucose levels

An excessive amount of fat in a meal slows down digestion and gastric emptying, thus making the insulin-carbohydrate association difficult to predict. Excessive fat favors the onset of hypoglycemia within the two hours following a meal and can delay hyperglycemia. Therefore, adequate food education is necessary to promote regular meal consumption and to learn how to count carbohydrates and calculate insulin doses. A suggestion for PWD and families is, therefore, to 1) follow their meal plan, 2) eat at least three evenly spaced meals each day with between-meal snacks as prescribed, and 3) plan meals no more than four to five hours apart. Families and caregivers of young PWD also need education on the risk factors for hypoglycemia so that they know when increased glucose monitoring is required and when treatment regimens need to be changed. Glucose monitoring using either flash or CGM should be performed before exercise, and extra carbohydrates may be consumed based on the glucose level and the expected exercise intensity and duration. Blood glucose targets may need to be adjusted upwards in children, adolescents, or young adults with diabetes with recurrent hypoglycemia and/or impaired hypoglycemia awareness.

7 Morbidity and mortality

7.1 Morbidity from hypoglycemia

Interest in the role of hypoglycemia as a cause of long-term morbidity has diminished. Exposure to chronic and repetitive hyperglycemia is now seen as a significant cause of permanent brain damage (64, 65). Transient cognitive dysfunction secondary to an episode of hypoglycemia is followed by a generally complete recovery within one hour of correction of low glucose levels. However, recovery from severe events can take up to 36 hours (66).

7.2 Mortality from hypoglycemia

Hypoglycemia has been proposed as a possible cause of "*death in bed*", which seems to be more frequent in children with T1D than in the healthy population (67). However, it is difficult to demonstrate a causal link between the hypoglycemic event and the cause of death; even the recent ISPAD hypoglycemia guidelines (1) attribute the fatal event mainly to arrhythmic causes, autonomic neuropathy, and genetic predisposition (68–70), not hypoglycemia *per se*. For these reasons, it is wiser to certify death as a concurrent series of causes that include hypoglycemia.

8 Fear of hypoglycemia and psychological impact

Fear of hypoglycemia (FoH) is a fear that affects the quality of life and diabetes outcomes in PWD (68). While adequate concern about hypoglycemia is functional for good glucose management, FoH is a specific and extreme fear evoked by the risk and/or occurrence of low blood glucose levels.

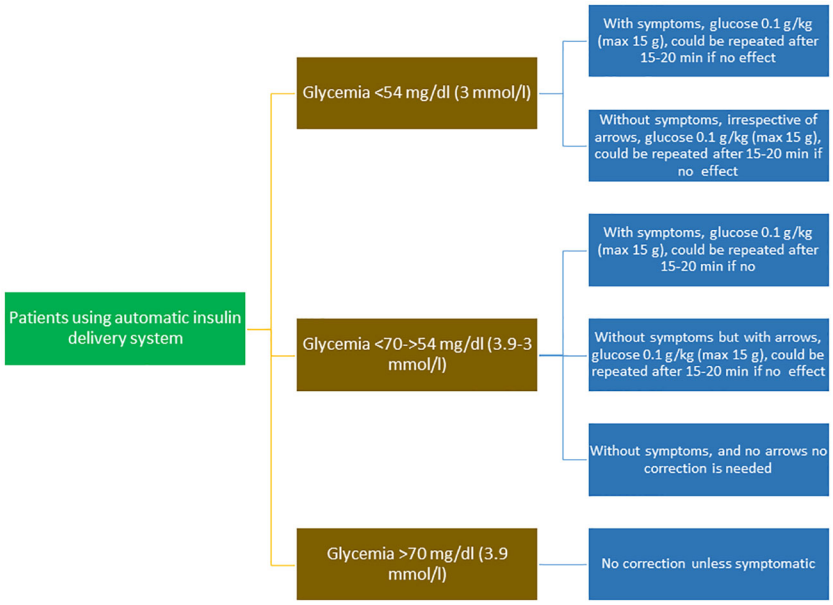

FIGURE 2
Flow chart of the treatment of hypoglycemic episodes in patients using automatic insulin delivery systems.

Despite hypoglycemia still being a significant impediment to glycemic control, especially in the pediatric age range, the inappropriate self-management of some PWD may be due to FoH rather than the hypoglycemia itself (71). Fear of hypoglycemia can lead to an excessive delay in the administration of boluses with negative effects on metabolic control (72). Furthermore, FoH reduces the propensity for physical activity (73).

FoH is a phenomenon that involves both parents and children. Frequently, subjects and caregivers attribute their high level of anxiety about hypoglycemia to previous severe hypoglycemic episodes (74). The impact of FoH on PWD manifests both at the psychological level and in glycemic control due to behavioral avoidance and affective distress. Symptoms of hypoglycemia can compromise social lives, and FoH can be so strong that PWD avoid social activities. Moreover, the potential risk of hypoglycemia can lead to excessive vigilance in glucose management, with high anxiety levels (75). Progressive and persistent increases in HbA1c after episodes of severe hypoglycemia have been described (76, 77). Routine screening for FoH is vital to identify those who would benefit from intervention (77).

8.1 Predictors of fear of hypoglycemia

While the strongest predictor of parental FoH is the experience of a severe hypoglycemic event with their child, fear can occur without previous hypoglycemia (77). FoH may be neither consciously perceived nor explicitly declared. Therefore, clinicians need to suspect and seek it out and have an especially high index of suspicion for "over-compensatory behaviors" (e.g., decreasing in insulin dosage or snacking), "avoidance behaviors" (e.g., limiting physical or social activities), acceptance of persistently high blood glucose levels, excessive daily blood glucose checks, and not implementing "agreed" treatment changes to lower blood glucose levels.

8.2 Measurement of FoH

A commonly used screening tool for FoH is the Hypoglycemia Fear Survey (HFS), which has been adapted for parents as the Hypoglycemia Fear Survey-Parents (78) and parents of young children as well as adolescents and children themselves (79, 80). The Children's Hypoglycemia Index (CHI) is another scale that has the added benefit of assessing FoH in specific situations such as only at night or school (80).

8.3 Fear of hypoglycemia and technologies

Advanced AID systems help to reduce nocturnal hypoglycemia, time spent in hypoglycemia, hyperglycemic episodes, and patient discomfort without increasing the risk of diabetic ketoacidosis or severe hypoglycemia (81). However, studies have not always reported greater satisfaction with treatment and a reduction in FoH with these systems (81). Patients who discontinued CGM showed a worsening of HbA1c levels compared with those who continued with CGM, who had a reduced FoH without improvements in glycemic levels (82). CGM systems with predictive alarms might reduce the time spent in hypoglycemia after physical activity (83), thereby contributing to the propensity for physical activity in PWD. Some believe that improvements in sleep quality and QoL in children and parents using these technologies are attributable to easier night-time control (84).

Finally, the use of advanced technologies result in significant improvements in parents' and children's sleep quality and in parents' FoH (85). The advancement and widespread deployment of such technologies has the potential to improve mental and physical health among PWD (86).

It is important to evaluate the psychosocial needs of young people with diabetes and their families when patients start using AID systems and during follow-up (87), establishing realistic expectations about the pros and cons of AID systems (86). Therefore, technological advances must be accompanied by well-timed training and adequate and continuous support (87, 88) and education.

8.4 Therapeutic interventions for FoH

Cognitive behavioral therapy and psychoeducational approaches have been shown to reduce this fear in adults. However, no studies have focused on children and adolescents, although these interventions may benefit older children (89). FoH contributes to the increased frequency of anxiety and depression in PWD and must be evaluated with a structured approach that includes specific screenings. Training must be individualized and take into account that those who have limited access to food due to cost also have limited options for dealing with hypoglycemia (90). A careful evaluation must also consider the coexistence of FoH in disadvantaged families (90).

The opinions of healthcare workers established using Delphi methodology indicated a need for specialized and expert staff (specialized nurses, educators) to train PWD on technologies applied to diabetes (91). Behavioral interventions for family members have shown persistent psychosocial benefits. CGM-focused education with behavioral support probably helps parents of young children with T1D reduce short- and long-term burden and worries (91). Furthermore, a telehealth approach may be helpful in the treatment of FoH (92).

9 Impaired awareness of hypoglycemia

Impaired awareness of hypoglycemia (IAH) is defined as the failure to perceive the appearance of autonomic warning symptoms and, consequently, the loss of ability to detect the onset of hypoglycemia and treat it promptly (93). IAH is associated with an approximately six-fold increased risk of developing severe hypoglycemia and represents a significant barrier to achieving optimal therapeutic goals. Four validated methods for assessing IAH are currently recognized: Clarke score, Gold score, Pedersen method, and HypoA-Q (94–97). Of these, the more detailed Clarke method seems to have higher specificity and accuracy for predicting the risk of clinically significant hypoglycemia (73) and, therefore, is preferred. CGM systems are also valuable tools for diagnosing IAH, especially if combined with one of the validated methods (98, 99).

According to epidemiological studies, the prevalence of IAH in children and adolescents with T1D assessed by the Clarke questionnaire varies from 16% to 22.4% (94, 100, 101). Younger age is the most reported factor associated with impaired hypoglycemia awareness (102–104).

IAH is also hypothesized to have a neurological component to its pathogenesis. Some brain regions, including the left amygdala and bilateral ventral striatum, show attenuated activation during hypoglycemic episodes, suggesting habituation of higher behavioral responses to hypoglycemia as a basis for unawareness (105). Recurrent hypoglycemia may be related to increased γ-aminobutyric acid inhibitory tone in the ventromedial hypothalamus and, thus, may be considered a mediator of hypoglycemia-associated autonomic failure (106, 107).

9.1 Restoring hypoglycemia unawareness

As the most critical risk factor for IAH is recurrent antecedent hypoglycemia, it is reasonable that the most crucial goal should be to reduce the incidence of hypoglycemia. Good metabolic control does not appear to increase the risk of unrecognized hypoglycemia, which is often associated with severe hypoglycemia and FoH (108).

Avoiding hypoglycemia interrupts the vicious cycle that impairs the ability of the adrenal medulla to produce epinephrine (a minor component of the counterregulatory response to hypoglycemia, which is primarily due to sympathetic neural activation) in response to blood glucose levels, restoring hypoglycemia awareness (109).

Technology can also help to restore hypoglycemia awareness; indeed, CGM is associated with a significant reduction in time spent in hypoglycemia episodes. Moreover, stopping insulin infusions when a low blood glucose value is encountered helps to avoid hypoglycemia and to re-start hypoglycemic symptoms. However, an unexpected limitation to restoring hypoglycemia awareness is that adolescents show a high acoustic arousal threshold from sleep (98), so they commonly continue to sleep through an alarm. Structured education on insulin administration, hypoglycemia training, blood glucose targets, and exercise management have also been shown to improve awareness of hypoglycemia (110–112).

10 Executive summary and recommendations

This document provides a series of clinical recommendations to prevent and treat hypoglycemia in children and adolescents with diabetes. All authors have long experience in the specialty and are members of the ISPED.

Overview

- Insulin-induced hypoglycemia and fear of hypoglycemia (FoH) are major limiting factors in glycemic management and a significant concern for children and adolescents with diabetes and their caregivers.

- Hypoglycemia is defined by autonomic or neuroglycopenic symptoms, low plasma glucose levels (<70 mg/dL), and symptomatic response to carbohydrate administration.
- Symptoms of hypoglycemia result from adrenergic activation (palpitations, sweating, shaking sensation) and neuroglycopenia (headache, drowsiness, difficulty concentrating). Younger children may exhibit behavioral changes such as irritability, restlessness, calmness, and tantrums.
- Three clinical levels of hypoglycemia are recognized:
  - ○ Level 1 – Clinical hypoglycemia alert

  A glucose value of <3.9 mmol/L (70 mg/dL) is an alert value that requires attention to prevent more severe hypoglycemia. The alert can be used as the threshold value for identifying and treating hypoglycemia in children with diabetes due to the potential for glucose levels to drop further.
  - ○ Level 2 - Clinically important or severe hypoglycemia

  Glucose values <3.0 mmol/L (54 mg/dL) indicate clinically significant or serious hypoglycemia. These low levels may lead to defective hormonal counter-regulation and impaired awareness of hypoglycemia (IAH). Neurogenic symptoms and cognitive dysfunction occur below this level, with a subsequent increased risk of severe hypoglycemia.
  - ○ Level 3 – Severe hypoglycemia

  Severe hypoglycemia is an event associated with severe cognitive impairment (including loss of consciousness and seizures) that requires the assistance of another person to administer intravenous carbohydrates, glucagon, or glucose.
- Children with diabetes can experience impaired hypoglycemia awareness and, when present, it is associated with a significantly increased risk of severe hypoglycemia.
- There have been significant reductions in the incidence rates of severe hypoglycemia over the past two decades for several reasons, not least the introduction of insulin analogues, improved diabetes technologies, and improved hypoglycemia education.
- Younger children often exhibit non-specific and behavioral symptoms due to combined adrenergic and neuroglycopenic responses, so the observed signs are more important than symptoms.
- Transient cognitive dysfunction secondary to a hypoglycemic episode is usually followed by complete recovery within one hour of correction of low glucose levels. However, recovery from severe events can take up to 36 hours. There is currently no high-quality evidence on the impact of hypoglycemia on lifelong cognitive impairment.
- Currently, available technologies such as continuous glucose monitoring (CGM), predictive low glucose management (PLGM), and automated insulin delivery (AID) systems reduce the frequency and duration of hypoglycemic episodes.
- Modifiable hypoglycemic risk factors include the type of insulin treatment and doses, physical activity, diet and alcohol habits, and drug use or substance abuse.
- The risk of hypoglycemia is greater with aerobic than anaerobic exercise. Nighttime hypoglycemia following exercise is mainly due to depletion of glucose stores, impaired counter-regulatory hormone responses during sleep, and increased insulin sensitivity due to nighttime fasting.
- Celiac disease, Addison's disease, and hypothyroidism should be considered in children with unexplained hypoglycemia.

Management

- The goal of treatment is to raise blood glucose above 70 mg/dL and to prevent further decreases.
- Oral glucose at a dose of 0.3 g/kg is the preferred treatment for the conscious individual with blood glucose <70 mg/dL (3.9 mmol/L), although any form of carbohydrate containing glucose may be used. Blood sugar levels increase about twice as fast with glucose than with sucrose (Figure 1).
- In patients treated with AID systems, non-severe hypoglycemia does not always need to be corrected. Correction is only needed for a hypoglycemia alert in symptomatic patients or within two to three hours of a bolus with an excess of active insulin, and usually a glucose dose of 0.1 g/kg is enough to raise glycemia above a safe level (Figure 2).
- Severe hypoglycemia occurring at home or school should be treated immediately with subcutaneous or intranasal glucagon.
- To treat severe hypoglycemia in a hospital setting, intravenous glucose (recommended dose 0.2 g/kg) must be administered immediately to limit exposure to hypoglycemia. Highly concentrated glucose solutions (50%) or infusion rates >5 mg/kg/min should be avoided due to the risk of excessive rate of osmotic change and, consequently, hyperosmolar cerebral injury.

Prevention

- Hypoglycemia should be prevented, as it is associated with severe physical and psychological distress in both patients and caregivers.
- Education and diabetes technologies are the primary tools for preventing hypoglycemia.
- Extending diabetes education to parents, schoolteachers, and other health professionals is a priority so that they can recognize early warning signs of hypoglycemia and treat low blood glucose immediately and appropriately.
- Glucose monitoring should be performed before physical activity, and carbohydrate correction should be performed as needed. Oral glucose should always be available during exercise.
- Adjusting insulin doses and changing glycemic targets may be necessary in children with frequent hypoglycemia.
- For documented hypoglycemia without symptoms or impaired awareness of hypoglycemia, parents should contact their diabetes care team to review the care plan.
- Impaired awareness of hypoglycemia should be routinely tested in clinical practice. Clarke, Gold, or Pedersen-

Bjergaard scores are useful for assessing impaired awareness. Reductions in hypoglycemia episodes may reduce impaired awareness of hypoglycemia.
- All children and adolescents with diabetes should be prescribed intranasal or subcutaneous glucagon. Patients, parents, and caregivers must be trained in its use.
- Periodic screening of children and parents for FoH helps to identify cases where more educational intervention is needed.

Future directions

- It is important to identify gaps in the skills and self-efficacy of children and adolescents with T1D and their families, especially when from a different cultural, socioeconomic, or educational background together with other perceived enablers of, and barriers to, self-management in this population.
- Specific educational paths are essential to help these people to correctly manage, treat, and prevent hypoglycemia episodes.
- Diabetes healthcare stakeholders may consider strategies for regular educational reinforcement in patients to foster healthy coping with diabetes stress, exercise planning to avoid hypoglycemia, interpreting blood glucose patterns, and adjusting medications or foods to reach target blood glucose levels.
- Furthermore, designing interventions that capitalize on how to use relevant technological devices could enhance diabetes self-management.

Author contributions

SZ: Conceptualization, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. ST: Methodology, Supervision, Validation, Writing – review & editing, Data curation, Investigation. AS: Conceptualization, Data curation, Investigation, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RB: Conceptualization, Supervision, Validation, Writing – review & editing. MD: Data curation, Investigation, Writing – review & editing. RF: Data curation, Investigation, Writing – review & editing. DI: Data curation, Investigation, Writing – review & editing. LL: Data curation, Investigation, Writing – review & editing. EM: Data curation, Investigation, Writing – review & editing. SP: Data curation, Investigation, Writing – review & editing. CP: Data curation, Investigation, Writing – review & editing. IR: Data curation, Investigation, Writing – review & editing. NR: Data curation, Investigation, Writing – review & editing. AR: Data curation, Investigation, Writing – review & editing. CR: Data curation, Investigation, Writing – review & editing. GS: Data curation, Investigation, Writing – review & editing. SS: Data curation, Investigation, Writing – review & editing. RS: Data curation, Investigation, Writing – review & editing. AZ: Data curation, Investigation, Writing – review & editing. VC: Conceptualization, Data curation, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Should we routinely assess hypothalamic–pituitary–adrenal axis in pediatric patients with Prader–Willi syndrome?

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Background: It has been reported that central adrenal insufficiency (CAI) in pediatric patients (pts) with Prader–Willi syndrome (PWS) may be a potential cause of their sudden death. In addition, the risk of CAI may increase during treatment with recombinant human growth hormone (rhGH).

Objective: To prevent both over- and undertreatment with hydrocortisone, we evaluated the prevalence of CAI in a large multicenter cohort of pediatric pts with PWS analyzing adrenal response in the low-dose ACTH test (LDAT) and/or the glucagon stimulation test (GST) and reviewing the literature.

Methods: A total of 46 pts with PWS were enrolled to the study, including 34 treated with rhGH with a median dose of 0.21 mg/kg/week. LDAT was performed in 46 pts, and GST was carried out in 13 pts. Both tests were conducted in 11 pts. The tests began at 8:00 a.m. Hormones were measured by radioimmunoassays. Serum cortisol response >181.2 ng/mL (500 nmol/L) in LDAT and >199.3 ng/mL (550 nmol/L) in GST was considered a normal response. Additionally, cortisol response delta (the difference between baseline and baseline) >90 ng/mL and doubling/tripling of baseline cortisol were considered indicators of normal adrenal reserve.

Results: Three GSTs were not diagnostic (no hypoglycemia obtained). LDAT results suggested CAI in four pts, but in two out of four pts, and CAI was excluded in GST. GST results suggested CAI in only one patient, but it was excluded in LDAT. Therefore, CAI was diagnosed in 2/46 pts (4.3%), 1 treated and 1 untreated with rhGH, with the highest cortisol values of 162 and 175 ng/dL, but only in one test. However, in one of them, the cortisol delta response was >90 ng/mL and peak cortisol was more than tripled from baseline. Finally, CAI was diagnosed in one patient treated with rhGH (2.2%).

Conclusion: We present low prevalence of CAI in pediatric pts with PWS according to the latest literature. Therefore, we do not recommend to routinely screen the function of the hypothalamic–pituitary–adrenal axis (HPAA) in all pts with PWS, both treated and untreated with rhGH. According to a review of the literature, signs and symptoms or low morning ACTH levels suggestive of CAI require urgent and appropriate diagnosis of HPAA by stimulation test. Our data indicate that the diagnosis of CAI should be confirmed by at least two tests to prevent overtreatment with hydrocortisone.

KEYWORDS

Prader-Willi syndrome (PWS), hypothalamic-pituitary-adrenal axis (HPAA), central adrenal insufficiency (CAI), low-dose ACTH test (LDAT), glugacon stimulation test (GST)

Our study presents the low prevalence of central adrenal insufficiency (CAI) in a multicenter cohort of pediatric patients with Prader–Willi syndrome (PWS) and summarizes 15 years of research on this issue. A detailed analysis of the results allows us to unequivocally exclude the need for routine diagnostics for CAI in this group of patients. The results of our work indicate that none of the diagnostic tests are sensitive enough to detect CAI, so the suspicion of CAI based on the result of one test should be confirmed in another. The prevalence of CAI is low in PWS and does not appear to increase with the introduction of growth hormone therapy. The diagnosis should be performed in patients with signs and symptoms suggestive of CAI and low morning ACTH levels.

Introduction

Prader–Willi syndrome (PWS) is a recognizable pattern of physical impairment with significant cognitive, neurological, endocrine, and behavioral abnormalities caused by a lack of gene expression from the impaired region inherited from the father of chromosome 15q11-q13, near the centromere (1). Patients with PWS develop hypothalamic dysfunction that can lead to various endocrine disorders (2). Besides the well-known endocrine abnormalities such as GH deficiency, hypogonadism, or pathological obesity, some studies over the last 15 years revealed the possibility of central adrenal insufficiency (CAI), the prevalence of which has been variously described in pediatric patients with PWS. These clinical observations were inspired by a study from 2008 by de Lind van Wijngaarden et al., which found that 60% of patients with PWS present CAI diagnosed on the basis of the test with metyrapone (3). Subsequent studies in 2010 showed a significantly lower prevalence of CAI (4%) based on the ACTH test and the insulin test (4) or did not confirm the presence of CAI in pediatric patients with PWS based on the ACTH test (5). Further studies also did not confirm such a high prevalence of CAI in patients with PWS (6–8). In the study by Corrias et al., CAI was confirmed in 14.3% of patients with PWS based on the low-dose ACTH test (LDAT) and in 4.8% of patients with PWS based on the standard-dose ACTH test (SDAT) (6). The latest studies in pediatric populations excluded CAI in patients with PWS based on the test with insulin (7) and based on the test with metyrapone, but not LDAT (8). Thus, the question "can CAI be part of the PWS phenotype in children?" still remains unanswered.

In addition, it is hypothesized that the risk of CAI may increase during rhGH treatment and may be a potential cause of sudden death in patients with PWS. GH inhibits 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), resulting in decreased conversion of cortisone to active cortisol. Therefore, it was postulated that patients with PWS treated with rhGH should be monitored for the possibility of an episode of adrenal insufficiency (2). The next question that needs to be answered is: Should the hypothalamic–pituitary–adrenal axis (HPAA) be routinely evaluated in all patients with PWS treated with rhGH? There is also no agreement on the prevalence of CAI in adults with PWS (9, 10). In some countries, it has been common practice to prescribe stress-dose hydrocortisone during physical or psychological stress in patients with PWS (6, 11). The side effects of frequent use of hydrocortisone include weight gain, osteoporosis, diabetes, and hypertension—already serious problems in adults with PWS. However, inadequate treatment of CAI can cause significant morbidity and even mortality. The most recent data from 2020 indicate that CAI is rare (1.2%) in adults with PWS (12). Based on multicenter data obtained from 82 patients, the authors advise against routine prescribing stress doses of hydrocortisone in adults with PWS. Therefore, it is time to ask again, what are the accurate and true data on CAI in children with PWS?

To prevent both over- and undertreatment with hydrocortisone in children with PWS, we evaluated adrenal response in LDAT and/or GST in a large multicenter cohort of pediatric patients with PWS. The aim of the study was to assess HPAA in patients with PWS (1) to obtain information about the prevalence of CAI in patients with PWS (2), to compare the results of HPAA diagnostic tests (3), to compare the results obtained in patients with PWS treated and untreated with

rhGH, and (4) to analyze the results with the currently available literature in order to propose common recommendations for the assessment of HPAA in patients with PWS.

Material and methods

Patients

We conducted a prospective cohort study. A total of 46 consecutive patients with PWS were enrolled in the study between 2014 and 2023, first in Krakow, and then, from 2021, in Warsaw and Szczecin. Thirty-four of them (74%) were treated with rhGH, and 12 were managed without rhGH treatment (before its introduction or because of severe obesity). We did not plan a control group. The study was approved by the Ethics Committee of the Jagiellonian University (consent number: KBET/212/B/2012 of 28 June 2012 and its updates of 4 April 2023, 27 September 2017, and 23 January 2020). All participants over the age of 16 and their parents have given written informed consent. Inclusion criteria were genetic confirmation of PWS by genetic testing, and the exclusion criteria were as follows: lack of parental consent to the study and acute infection at the time of the study (in these cases, the tests were postponed). In patients on steroid therapy, including inhaled steroid therapy, tests were performed after discontinuation of steroids after the time dependent on the steroid used. The clinical characteristics of patients with PWS are presented in Table 1.

Methods

After an initial medical history and physical examination, HPAA assessment was performed on all enrolled patients using LDAT and/or GST. The aim of the project was to perform both tests in all patients in order to diagnose HPAA and compare the tests. It was not possible to achieve this assumption in all patients due to the temporary lack of synthetic ACTH or the lack of consent of the patients' parents for another test, when the first one ruled out adrenal insufficiency.

Low-dose ACTH test

The patient was installed with a venous sampling catheter early in the morning around 7:00–7:30. At 8:00 a.m., synthetic ACTH (tetracosactide) was administered intravenously at an absolute dose of 1 μg or 0.5 $\mu g/m^2$ of body surface area. Serum cortisol and plasma ACTH were measured at baseline, then cortisol levels were measured 20, 30, and 60 min after ACTH administration.

Glucagon stimulation test

The installation of the catheter for venous sample collection was done in the same way as in LDAT. Around 8:00 a.m., glucagon was administered intramuscularly at a dose of 0.1 mg/kg (maximum dose of 1.0 mg) (12). Plasma ACTH and serum cortisol were measured at baseline. Blood glucose levels were measured at baseline and every 30 min up to 180 min after glucagon injection. In addition, blood for serum cortisol measurement was drawn every 30 min from 90 min to 180 min after glucagon injection, at the time of expected onset of hypoglycemia.

Normal ranges and interpretation of tests

Serum cortisol response >181.2 ng/mL (500 nmol/L) in LDAT and >199.3 ng/mL (550 nmol/L) in GST was considered a normal response. In addition, the cortisol response delta (the difference between baseline and its highest value) >90 ng/mL or doubling/tripling of baseline cortisol indicates normal adrenal reserve. The normal range for morning ACTH is 10–60 pg/mL, and that for morning cortisol is 50–230 ng/mL (13–17).

Biochemical methods

Plasma ACTH and serum cortisol levels were measured by radioimmunoassays according to the instructions of their producers (ACTH—Brahms, Germany and cortisol—Siemens Healthcare Diagnostics, USA).

Statistical analysis

The results were statistically analyzed using the Dell Statistica 13.1 64-bit package (StatSoft, Poland, Kraków). Results are presented as a median (95% confidence interval) or as a mean ± SD or as %. To test if the variance in cortisol levels was different between the two groups (patients with PWS treated and untreated with rhGH) and between the two stimulation tests (ITT and GT),

TABLE 1 Clinical characteristics of patients with Prader–Willi syndrome.

Pts with PWS	N	Female	Male	Mean age (years)	Mean bone age (years)	Mean height (SD)	Mean BMI (SD)	Mean rhGH dose (mg/kg/week)	Median IGF-1 (SD)	HbA1c (%)
Treated with rhGH	34	16	18	9.5	9.7	−0.30	2.30	0.21	1.8	5.3
Untreated with rhGH	12	6	6	11.7	11.9	−2.84	2.58	–	−1.6	5.4

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analysis of variance was used. A *p*-value of less than 0.05 was considered statistically significant.

Results

We conducted a total of 58 tests: 45 LDATs and 13 GSTs. Both tests were performed on 11 patients. We did not observe any serious side effects from either test. Three GSTs were not diagnostic (no hypoglycemia was achieved). Two patients had LDATs twice, due to repeated low cortisol levels in the morning without a clinical CAI picture.

LDAT results suggested CAI in four patients, but in two out of four patients, CAI was excluded at GST. GST suggested CAI in only one patient, but was excluded in LDAT. Therefore, CAI was diagnosed in 2/46 patients (4.3%), 1 treated and 1 untreated with rhGH, with the highest cortisol values of 162 and 175 ng/dL, but only on the basis of one test. However, in one of them, the cortisol delta response was >90 ng/mL and more than tripled from baseline. Finally, CAI was diagnosed in one PWS patient treated with rhGH (2.2%), but only on the basis of LDAT. The results of the diagnostic tests are presented in Table 2.

Morning/basal cortisol levels were within the normal range in all patients with PWS not treated with rhGH. Low morning/basal cortisol levels were observed in 4 of the 34 patients treated with rhGH (12%); however, in all of these cases, CAI was excluded by dynamic testing. In contrast, the only CAI patient diagnosed only by LDAT had normal morning cortisol. All patients, both treated and untreated with rhGH, had normal morning ACTH levels. None of our patients developed signs and symptoms of adrenal insufficiency, and none of them were treated with hydrocortisone on a permanent basis. In our only patient with CAI, we recommend treatment in a stressful situation, and we plan to perform another HPAA assessment test.

We found no differences in morning ACTH and cortisol values, peak cortisol and cortisol delta between LDAT and GST, and between patients with PWS treated and untreated with rhGH.

Discussion

The results of our study confirmed a very low prevalence of CAI in patients with PWS, consistent with most previous references (Table 3). Data reports previously from different clinical centers differed even when the same diagnostic tests were used to assess HPAA. The key issue seems to be the correct diagnosis of CAI in this group of patients. It is well known that CAI cannot be determined in patients with PWS by measuring cortisol levels under stressful conditions such as high fever, as patients with PWS may not present with fever during severe infection due to hypothalamic disorders. Therefore, a stimulation test may be the best way to detect adrenal insufficiency. There are several tests used for CAI detection: LDAT, metyrapone test, insulin-induced hypoglycemia test (IIHT), glucagon stimulation test (GST), and corticotrophin hormone (CRH) stimulation test (13, 14). Which test is the best? The IIHT is the gold standard for evaluating the presence of CAI, but it can be dangerous especially in patients with PWS due to the possibility of decreased count regulation hormone response. Metyrapone testing can induce an acute adrenal crisis in patients with a chronic glucocorticoid deficiency and induces sensations such as nausea, vomiting, and hypotension—symptoms of cortisol deficiency also in healthy individuals. The results of the CRH stimulation test, which evaluates the ability of the pituitary gland to secrete ACTH to stimulate cortisol production, are difficult to interpret. There are conflicting reports regarding the cutoff points for this test, and furthermore, the availability of CRH is problematic in some countries. LDAT has high sensitivity in the diagnosis of adrenal dysfunction, but may miss benign CAI (15, 16). The GST

TABLE 2 Results of diagnostic tests for CAI in patients with PWS. Median and ranges are presented. LDAT, low-dose ACTH test; GST, glucagon stimulation test.

Tests	Basal ACTH (pg/mL), normal range, 10–60 pg/mL	Basal cortisol (ng/mL), normal range, 50–230 ng/mL	Peak cortisol (ng/mL)	Delta of cortisol in test (ng/mL)	No. of tests with doubling/triplication of baseline cortisol
LDAT in treated with rhGH	10.2–61.3 28.1	34.6–190.4 93.5	162–355.8 245.6	64.3–287.3 152.4	30/33
LDAT in untreated with rhGH	22.2–24.9 23.5	43.5–159.7 82.5	165.4–316.8 223.3	75–187.9 117.7	8/10
GST in treated with rhGH	10.2–45.5 35	55.3–179.4 73.9	161.2–312.5 246.6	93.6–246.1 175.8	7/8
GST in untreated with rhGH	15.2–37.3 26.8	54–73.4 63.7	233.9–245.3 239.2	179.9–209.8 194.9	2/2
LDAT	10.2–61.3 27.5	34.6–190.4 79.0	162–355.8 235.2	64.3–287.3 152.2	38/43
GST	10.2–45.5 29.5	54–179.4 80.4	161.2–312.5 236.0	93.6–246.1 179.9	9/10

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TABLE 3 Results of studies assessing the prevalence of central adrenal insufficiency in pediatric patients with Willi–Prader syndrome.

Study	*N*	Median age (range)/mean age (SD)*	Testing method	Cutoff points in tests	Treated with GH/ untreated (%)	Prevalence of CAH (%)
de Lind van Wijngaarde et al. (3)	25	9.7 (3.7–18.6)	MT	11-DOC >200 nmol/L (7 µg/dL)	25/0 (100%)	**60**
Connell et al. (4)	4 6 15	7.16 (0.43–16.27)	LDAT SDAT IIHT	Cortisol >18 µg/dL (500 nmol/L) for all tests	Lack of data	**4** **(1 pt in IIHT)**
Nyunt et al. (5)	41	7.68 (± 5.23)*	LDAT	Cortisol >18 µg/dL (500 nmol/L) for all tests	19/22 (46%)	**0**
Corrias et al. (6)	84 (9 had both tests)	7.7 (± 5.0)*	LDAT HDST	Cortisol >18 µg/dL (500 nmol/L)	53/31 (63%)	**14.2** **4.8**
Beauloye et al. (15)	20: 14 7 (1 had both test)	4.55 (0.8–14.7) 5.6 (3.5–14.4)	GST IIHT	Cortisol >20 µg/dL (550 nmol/L) and/or delta cortisol* >9.0 µg/dL (250 nmol/L) for both tests	5/15 (25%)	**5/0***
Obrynba et al. (8)	21	13.9 (± 10.9)*	LDAT MT	Cortisol ≥15.5 µg/dL (428 nmol/L) 11-DOC >200 nmol/L (7 µg/dL)	16/5 (76%)	**29** **0**
Oto et al. (7)	36	2.0 (0.6–12.0)	IIHT	Cortisol >18 µg/dL (500 nmol/L)	0/36 (0%)	**0**
Angulo et al. (18)	128	8.63 (± 5.3)* (0.3–18)	No test, only morning ACTH and cortisol	Normal ranges: Cortisol 3–25 µg/dL (83–690 nmol/L) ACTH 9–57 pg/mL (2–13 pmol/L)	128/0 (100%)	**0**
Grootjen et al. (19)	93 (30 had two times) (11 had three times) (1 had four times)	0.97 (0.48–4.81)	MT	ACTH peak >13 pmol/L (59 pg/mL) 11-DOC >200 nmol/L (7 µg/dL)	75/18 (76%) 30/0 (100%) 11/0 (100%)	**0**
Our study	46: 45 (2 patients had two times) 13 (11 had both tests)		LDAT GST	>181.2 ng/mL (500 nmol/L) >199.3 ng/mL (550 nmol/L) and/or delta cortisol >9.0 µg/dL (250 nmol/L) or doubling/triplication of baseline cortisol for both tests	34/46 (74%)	**2.2**

CAI, central adrenal insufficiency; MT, metyrapone test; LDAT, low-dose ACTH test; HDAT, high-dose ACTH test; IIHT, insulin-induced hypoglycemia test; GST, glucagon stimulation test; delta cortisol—an increase of cortisol calculated between the lowest cortisol and the highest cortisol level during the stimulation test.
*-mean age (SD).

has the same sensitivity as LDAT for diagnosing CAI, but may pick up more subtle abnormalities of the HPAA. It is considered an equal and safe alternative to the IIHT and produces similar cortisol responses. An obtained hypoglycemia in both tests is a fast and potent stimulus of CRH secretion, which will, in turn, enhance ACTH secretion and thereafter cortisol production. Thus, a normal response to hypoglycemia requires integrity of the entire HPAA (14).

The highest incidence of CAI was reported by the Dutch team in 2018 (3). The authors performed only one test with metyrapone in the diagnosis of CAI. All of their patients underwent this examination during an overnight stay in the Pediatric Intensive Care Unit. Further studies have shown a significantly lower incidence of CAI or even its absence in patients with PWS (4–8). Recent data indicate that CAI is not a crucial clinical problem in PWS, but these patients do have a delayed HPAA response during

acute stress (7). In the study by Oto et al., basal and peak cortisol levels were within the normal range, but the peak cortisol response to ITT was delayed in the majority of patients with PWS (64%) (7). Although the mechanism remains unclear, this delay may indicate the existence of a central obstacle to HPAA adjustment. This delayed HPAA response during acute stress has also been reported in a recent study by Grootjen et al. (19). Perhaps in the study by Wijngarden et al., delayed adrenal response was not included in the final results (3). In contrast, in the study by Obrynba et al., CAI was excluded in all 21 participants with PWS using the metyrapone test, although 29% of them simultaneously failed LDAT (8). Analyzing the results of this study, it is worth noting that the authors started LDATs in all patients at 10:00 p.m. This is the period of the day when cortisol levels are at their lowest, which is why such a high incidence of CAI can be a false-negative test result. Moreover, after the LDAT was completed, metyrapone was administered at midnight on the same day. The adrenal glands were "primed" with ACTH, and this may be the reason why the cortisol response in this test with metyrapone was not delayed (8). Grootjen et al., who also used the test with metyrapone, excluded CAI in all patients with PWS (19). However, in almost 30% of their patients, the authors repeated tests. In some patients, they even repeated the test more the twice. In all studies that used the metyrapone test, the majority of patients were treated with rhGH. Overall, two of the three studies diagnosing HPAA in patients with PWS using the test with metyrapone excluded CAI, and one indicated a high incidence of CAI. LDAT was used to diagnose HPAA in patients with PWS in five studies. In two of them, it allowed the exclusion of CAI (4, 5). In one analyzed previously, the incidence of CAI was 29% but most likely due to false-positive results (8). In the study by Corrias et al., LDAT indicated a CAI prevalence of 14.2%, but it was verified with the standard ACTH test and the prevalence dropped to 4.8% (6). However, there are opinions that the standard ACTH test is not a good tool in the diagnostics of CAI (14), and there is a report indicating that both the standard test and LDAT had a similar diagnostic accuracy in adults and children using different peak serum cortisol cutoff values (16). In our study, LDAT allowed for the diagnosis of CAI in one patient (2.2% of all patients with PWS studied), but it was not confirmed by another test. HIIT was used in three studies, and two of them excluded CAH in all patients with PWS (7, 17), while one study reported a low 6% prevalence of CAI but was not verified by another test (4). In a study by Beauloye et al., CAI was suspected based on an insufficient peak cortisol value of 16.6 µg/dL after insulin injection, but this patient's cortisol delta was 9.97 µg/dL and CAI was eventually ruled out (15). Two studies used GST in the diagnostics of HPAA, and in both studies, CAI was excluded (17).

Untreated GH deficiency may mask CAI. Low insulin-like growth factor 1 (IGF-1) levels result in increased expression and activity of 11β-HSD1, the enzyme that converts cortisone to cortisol (20). Our study, or none of the previously cited studies, is unlikely to confirm a strong association between CAI prevalence and rhGH treatment. Studies assessing HPAA in patients with PWS untreated with rhGH or treated in the minority did not report cases with CAI (5, 7, 17). When patients were treated with rhGH, the prevalence of CAH amounted to 0%–60%; however, only in one study was this prevalence high—60% (3); in was low in two studies: 2.2% (our data) and 4.8% (6), and three studies found no evidence of CAI (8, 18, 19).

Based on our data and a review of the literature on the incidence of CAI in children and adolescents with PWS, we do not recommend a routine diagnostics of HPAA in this group of patients. If the clinical picture suggests CAI in patients with PWS, an appropriate test should be used and confirmed with another test. Our study, similar to that of Obrynda et al. and Grootjen et al., clearly indicates that the suspicion of CAI in one test should be verified by another test (8, 19). It is known that cortisol levels in a stressful situation are not a good diagnostic tool in the diagnosis of HPAA in patients with PWS. However, based on the results of recent data published by Angulo et al., morning ACTH and cortisol levels may be reliable and helpful in monitoring HPAA function (18). The authors found that patients with PWS had significantly lower morning cortisol levels than the control group, although in both groups, morning cortisol and ACTH levels were in the normal range. Our study did not confirm this observation because in 12% of our patients with PWS treated with rhGH, morning cortisol levels were below normal, but tests ruled out CAI in all of them. In contrast, the only patient with CAI confirmation in single LDAT had normal morning cortisol levels. Since all of our patients with WPS treated and untreated with rhGH had normal morning ACTH levels, it was the morning ACTH level that appears to be more reliable for CAH screening in this group of patients.

Conclusions

The low incidence of CAI in pediatric patients with PWS is presented according to the latest literature. Therefore, routine screening of HPAA function is not recommended for all patients with PWS, both treated and untreated with rhGH. According to the review of the literature, signs and symptoms or low morning ACTH levels suggestive of CAI require urgent and appropriate diagnosis of HPAA by stimulation test. Our data indicate that the diagnosis of CAI should be confirmed by at least two tests to prevent overtreatment with hydrocortisone.

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 humans were approved by the Ethics Committee of the Jagiellonian University (consent number: KBET/212/B/2012 of 28.06.2012 and its updates of 4.04.2023, 27.09.2017 and 23.01.2020). 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

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Conflict of interest

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Healthcare professionals' perspectives towards the digitalisation of paediatric growth hormone therapies: expert panels in Italy and Korea

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Introduction: To analyse the perspectives of healthcare professionals (HCPs) regarding the acceptance of digital health solutions for growth hormone (GH) deficiency care. This study identified factors impacting HCPs' intent to use and recommend digital solutions supporting recombinant-human growth hormone (r-hGH) therapy in Italy and Korea with a use case of connected drug delivery system (Aluetta® with Smartdot™) integrated in a platform for GH treatment support (the Growzen™ digital health ecosystem).

Methods: Participatory workshops were conducted in Rome, Italy, and Seoul, Korea, to collect the perspectives of 22 HCPs on various predefined topics. HCPs were divided into two teams, each moderated by a facilitator. The workshops progressed in five phases: introduction of the project and experts, capturing views on the current context of digitalisation, perceived usefulness and ease of use of Aluetta® with Smartdot™, exploration of the perception of health technology evolution, and combined team recommendations. Data shared by HCPs on

technology acceptance were independently analysed using thematic analysis, and relevant findings were shared and validated with experts.

Results: HCPs from both Italy and Korea perceived Aluetta® with Smartdot™ and the Growzen™ based digital health ecosystem as user-friendly, intuitive, and easy-to-use solutions. These solutions can result in increased adherence, a cost-effective healthcare system, and medication self-management. Although technology adoption and readiness may vary across countries, it was agreed that using digital solutions tailored to the needs of users may help in data-driven clinical decisions and strengthen HCP–patient relationships.

Conclusion: HCPs' perspectives on the digitalisation in paediatric GH therapies suggested that digital solutions enable automatic, real-time injection data transmission to support adherence monitoring and evidence-based therapy, strengthen HCP–patient relationships, and empower patients throughout the GH treatment process.

KEYWORDS

adherence monitoring, Aluetta®Smartdot™, connected device, digital health, healthcare digitalisation, recombinant human growth hormone

1 Introduction

Digital health technologies and the use of connected devices are progressing rapidly, becoming an integral element of healthcare delivery (1, 2). Digital health technologies have paved the way for enhanced patient care and management of chronic conditions, especially with the advent of connected devices that facilitate the capture of objective data about patients (2). The global 5-year Easypod Connect Observational Study suggest that connected digital devices can significantly improve patient outcomes for recombinant human growth hormone (r-hGH) therapy in children with growth failure (3, 4), thereby enhancing patient adherence. They also improve therapeutic monitoring and patient support provided by healthcare professionals (HCPs) (5–7), which is a key step in the patient's care pathway (8, 9). Adoption of digital health solutions by HCPs is correlated with desired outcomes, warranting the need to understand the attitudes of HCPs towards prescribing their use (9). HCPs remain at the forefront of creating awareness, motivating, and providing family-centered, personalised care and management; hence, adoption of digital solutions requires participatory assessment of their perceptive (5–7). Understanding factors associated with willingness of HCPs to prescribe digital health solutions to patients is important (6, 8, 10–14). However, limited information exists regarding barriers and enablers for the use of digital health ecosystems in long-term paediatric care (15).

An example of a digital health ecosystem supporting the monitoring and self-management of patients with growth hormone deficiency (GHD) is the Growzen™ digital health ecosystem. This solution includes Aluetta® with Smartdot™, a novel digitally connected, reusable, multi-dose injection pen device for administering r-hGH (Saizen®, Merck KGaA, Darmstadt, Germany). Incorporating a smart attachment for data transmission, this innovative adherence sensor-based device combines the ease of use of the Aluetta® manual pen with advanced capabilities, and its integration with a digital health ecosystem empowers HCPs with remote monitoring of patient adherence, enabling timely intervention and decision-making (16). This ecosystem currently includes Aluetta® with Smartdot™, Growzen™ Buddy [a mobile app for patients and caregivers to guide them for growth hormone (GH) therapy] and Growzen™ Connect healthcare professional platform (used by HCPs to track treatment adherence and outcomes for GH patients) (Figure 1).

To assess the potential impact of digitalisation on the willingness of HCPs by integrating the connected GH injection pen into their clinical practice, participatory workshops involving an expert panel were conducted in Italy and Korea in 2022 to capture a broader view on acceptance of the solution across diverse healthcare ecosystems. This study aimed to understand the extent of acceptability and perceptive in countries with different level of readiness and cultural acceptance for a digital healthcare ecosystem enabled by the use of the connected injection pen for r-hGH. This qualitative study explored current attitudes towards the digitalisation of r-hGH therapy in the two countries through panel discussions, analysed HCPs' perceptions regarding potential acceptance of the connected device compared with other non-connected alternatives (e.g., pen and paper adherence diaries), and assessed factors affecting their intent to use and integrate digital health solutions supporting r-hGH therapy in clinical practice.

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FIGURE 1
Growzen™ digital health ecosystem. Aluetta® with Smartdot™ is used to provide growth hormone injections to patients and transmit real-time injection data. Growzen™ Buddy is a mobile app for patients and caregivers to guide them for growth hormone therapy. Growzen™ Connect healthcare professional platform is used by healthcare professionals to track treatment adherence and outcomes for growth hormone patients. HCPs, healthcare professionals.

2 Methods

2.1 Experts and locations

Participatory workshops were conducted in Italy and Korea to explore the perceptions of two expert panels on the acceptance of connected devices and technological evolution, considering Aluetta® with Smartdot™ within the Growzen™ digital health ecosystem as an example of digital solution. The workshops were spread over 4 h and conducted on 25 November 2022 in Rome, Italy, with eight HCPs (five paediatric endocrinologists and three endocrinologists) and on 2 December 2022 in Seoul, Republic of Korea, involving 14 paediatric endocrinologists. HCPs with experience in paediatric/transition/adult GHD treatment participated in the panels regardless of their previous digital health experience. Adult endocrinologists in Italy were asked to provide their opinion on patient care focussed on the transition from patients with childhood-onset GHD to adult patients.

2.2 Workshop structure, activities, and materials

Experts in each workshop were grouped into two teams to independently perform several activities based on professional expertise, age, and sex. Each team was moderated by a facilitator with experience in participatory methods. The two teams were initially together in the same room for introductions and explanations of the phases and tasks of the workshop and then performed these activities in separate rooms to capture information around perceptions and then finally combined for conclusive discussions and recommendations. Data from all experts were collated for qualitative analysis.

Sticky cards representing two contexts were provided to the experts to identify factors and share their opinions according to various predefined topics. The first context considered was technology acceptance encompassing self-administration, wherein the patients administered the therapy, although caregivers took care of patients' health and managed their treatments. The other context was the therapy administered by caregivers, wherein they took overall care of the health and therapy management with paediatric patients not being autonomous enough to be responsible for their own treatment.

A description of Aluetta® with Smartdot™ device within the Growzen™ digital health ecosystem was provided in an introductory video. Additionally, experts had the opportunity to see and touch the device during the session. During the workshop, experts were asked to provide their opinions on Aluetta® with Smartdot™ orally, and the session was audio recorded to complement the notes from the moderators. Experts were prompted by various predefined topics based on their clinical experience. Each workshop progressed in five phases (Figure 2).

The first phase comprised an introduction of the project and experts with a description of the workshop structure and concrete tasks and activities to be performed. In the second phase, the views on the current context of digitalisation were captured, and the experts provided opinions and comments about several predefined topics such as the importance of treatment adherence, perceived usefulness of collecting patients' adherence data, current methods used to collect adherence data, use of digital health tools with a focus on HCPs' experience in using these solutions in their daily clinical practice, and perspectives on the patients' attitudes towards the use of digital health tools. Experts identified factors related to three entities (patients/caregivers, healthcare centres, and HCPs) in the template and the relationship between these entities (care services, facilitating conditions, and HCP–patient relationships).

The third phase assessed the perceived utility and ease of use of Aluetta® with Smartdot™ device (as an example of a digital health solution) considering both the defined contexts. Following the concrete instructions provided by the moderator, preceded by the introductory video of the device, experts discussed and identified

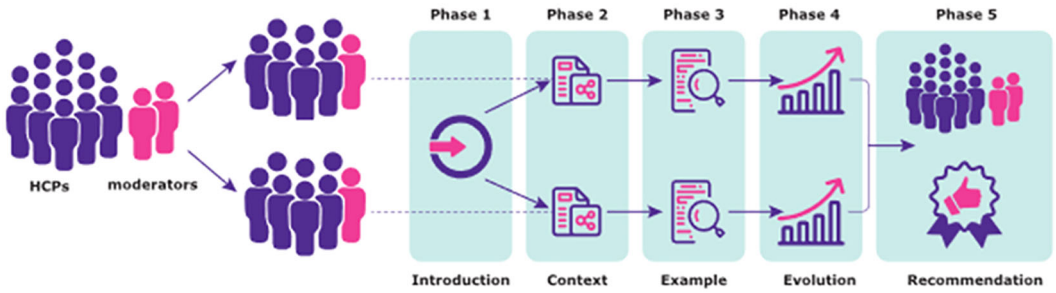

FIGURE 2
Workshop structure.

relevant issues, strengths, and weaknesses of the digital device in the context of GHD management in their respective countries. The experts were provided with predefined study cases, namely, new pen users and Aluetta® pen users (i.e., without the Smartdot™ attachment), with the lack of adherence to guide the discussions. A set of predefined topics were pursued that included relevant issues such as ergonomics, perceived ease to configure the device, perceived ease to use the device, perceived learnability, perceived ease to teach the configuration process, flexibility (removing Smartdot™), perceived usefulness for HCPs, perceived usefulness for patients, potential adoption for each study case, appropriateness for each study case, and potential risks associated with the use of the digital device.

In phases 2 and 3, predefined templates were given to the HCPs along with a set of sticky cards representing predefined topics to facilitate the activity. In the fourth phase, HCPs' perceptions on health technology evolution in paediatric/transition/adult GHD care were explored. Three scenarios representing different technological generations were introduced by the moderator. The first scenario represented the use of a pen without any digital capability and a manual diary to collect adherence data (non-digital alternative), whereas the second scenario consisted of the use of a pen without any digital capability and a mobile app to register adherence data through manual inputs (partially digital alternative). The third scenario represented the use of Aluetta® with Smartdot™ integrated in the Growzen™ digital health ecosystem to collect adherence data (fully digital alternative). Templates used in phase 3 represented these three scenarios and included topics related to the corresponding activity focussed on the adherence data collection process, potential impacts of each scenario on daily practice, and patient self-management considering both contexts. Experts were asked to identify the strengths and weaknesses of the scenarios regarding the discussed topic. Additionally, study cases defined in the previous phase were used to guide the discussions.

In the fifth and final phase, all experts were combined for team recommendations in one room. Each team briefly presented the identified factors and discussed them along with the most relevant findings on the use and recommendation of digital health solutions, in particular, Aluetta® with Smartdot™ in the current healthcare setting reported during the previous activities. The moderator asked experts to describe their opinions about the relevance of factors and summarised the conclusions reached in each session.

2.3 Data collection and analysis

The participatory workshop sessions were audio-recorded and reviewed by the facilitators. Thereafter, relevant comments were transcribed, and information from the facilitators' notes and text included in the predefined templates was collated. The data collected in this study were evaluated using a qualitative approach similar to that defined in the thematic analysis. Relevant findings were shared and validated with experts.

All procedures performed in this study were in accordance with European and national ethical guidelines, the European Code of Conduct for Integrity in Research, the Universal Declaration of Human Rights, and the Helsinki declaration. HCPs were informed about the research topic and procedures before joining the expert panel. The experts provided their opinions based on their experience on this topic and were not the main subjects of the study. The experts' opinions included as quotes were pseudo-anonymised. No sensitive information was used or collected, and the contributions of the expert panel had no impact on others.

ORR reviewed all collected data, coded them, and defined themes, after which all authors reviewed the proposed themes and refined them until consensus was reached.

3 Results

The results presented here describe HCPs' perspectives towards the digitalisation of growth hormone therapies and do not originate from scientific data. Overall findings suggest that each country health system and socio-economic and educational landscape may be varied, shaping the HCPs perspectives on the utility of digital health solutions and difficulty to seamlessly transfer from one setting to another. Four major themes were identified for presenting the data analysis: 1) understanding the context of digital transformation, 2) relevant digital health design considerations, 3) perceived benefits and risks of using digital solutions for adherence monitoring, and 4)

perceived usefulness and ease of use of Aluetta® with Smartdot™ and the Growzen™ digital health ecosystem.

3.1 Understanding the context of digital transformation

In this theme, experts' comments expressing their perspectives on how their organisations were supporting the use of digital health solutions, including adherence monitoring, were recorded. Stakeholders' perspectives on the use of novel solutions along with current strategies used to manage growth disorders were also considered.

3.1.1 Organisational and technical support

This subtheme collected experts' comments about support provided by organisations, healthcare institutions, and other entities for the use of digital health solutions towards management of growth disorders.

In both Korea and Italy, experts agreed on the importance of monitoring adherence to GH treatment. However, experts in Italy perceived that the use of digital health solutions to monitor adherence was not a priority for the Italian healthcare system due to the lack of involvement of health institutions. The potential benefits of such solutions were not considered in their evaluation. They mentioned that adherence is not often used in cost-effectiveness analysis.

In Italy, experts identified the pharmacoeconomic approach of the healthcare system while making decisions. Similarly, the Korean panel also reported some economic aspects, such as the fact that some GH treatments were financed by insurance companies that may lead to higher adherence, as suggested by academic reports. In these circumstances, monitoring adherence to treatment was mandatory, and caregivers were required to provide adherence data to physicians.

> *"In case of continuous glucose monitoring it is mandatory that a certain percentage of adherence is observed in order to receive reimbursement (insurance coverage) from the government. So, caregivers are obliged to show us the data and doctors also check it thoroughly and make data entry." [Korea]*

While Korean HCPs could perceive the use of digital health solutions as an additional task to perform in their existing short visit time, they mentioned extrinsic motivation, such as payment of fees, as a relevant factor, whereas the Italian panel emphasised the role of the healthcare institutions in convincing and supporting HCPs in integrating these solutions (additional bureaucracy, training, and clear guidelines, etc.). Both the panels agreed on the need for training and some external services to support them in their tasks (for example, patient support programmes).

> *"I would prefer a course. It's not difficult, but I would put the need for targeted education" [Italy]*

3.1.2 Perspectives on the use of a digital health solution

The Korean panel considered the Korean society to be highly digitalised and, therefore, thought that HCPs may have a positive outlook towards the use of digital health solutions. They discussed the need to tailor digital solutions to patients and caregivers and emphasised that these solutions should be user-friendly.

> *"Nowadays, most people are very used to smartphones and digital applications. So, I don't think that there will be any significant barriers in using this kind of application. But making it user-friendly, I think, is very important." [Korea]*

Conversely, Italian experts reported that some HCPs might not be interested in the collected adherence data. Consequently, they may not promote digital health solutions due to lack of interest perceiving an increased workload and wastage of time. However, they agreed that having trustworthy data and accessing them through a usable platform would be beneficial for patient management.

> *"Because this trust in having a useful device for patient management, and the attitude of trust even in regard to rapidly usable data." [Italy]*

Both Korean and Italian panels highlighted the need to integrate digital health solutions into clinical practice. Additionally, the experts commented that this integration should be performed gradually, allowing patients/caregivers to use preferred tools for adherence monitoring. Although both panels expressed the need for actionable recommendations based on the collected data, the Korean panel preferred a brief paper summary to be used in their visits despite the highly digitalised context. However, the Italian panel agreed that they would like to receive these feedback and recommendations through a digital platform such as Growzen™ Connect HCP platform.

> *"Perhaps it is not used in daily life, because there is not a moment that comes to mind during the visit to say I'm going to check this thing." [Italy]*

Regarding patients'/caregivers' perspectives on the use of a digital health solution to support GHD management, the Italian panel focussed on individual characteristics such as age, digital literacy, and response to external control. Although the Korean panel also identified some individual characteristics, they focussed on technical aspects. For example, they commented on the importance of encouraging patients to continue using digital health solutions for a longer duration.

Both Korean and Italian experts agreed that patients/caregivers should be aware of the potential benefits for the management of the condition of using the digital health solution. This approach was considered a key strategy to convince experts/caregivers to adopt digital health solutions.

> *"What do you think about users' attitude towards using the mobile APP? Initially reluctant, it is important to motivate them to use and explain the importance of adherence." [Italy]*

3.1.3 Managing growth disorders

Both countries reported several strategies to monitor patient adherence. Most Korean experts commented that they asked about adherence or the number of missed injections to patients or caregivers. Similarly, the current adherence data collection strategies in Italy were based on collecting subjective data, leading to unreliable datasets. HCPs also reported their concerns about the accuracy of collected data with non-digital entries.

> *"It is well known that adherence is related to treatment efficacy, and most caregivers understand that their children need regular injections to be able to expect a good treatment outcome. This then leads to the question of 'how can we improve adherence?'" [Korea]*
> *"In the case of written data (e.g. diary), they are difficult to analyse, require enormous expenditure of time on the part of the doctor, risk of error and lack of objectivity of the data" [Italy]*

3.2 Relevant digital health design considerations

3.2.1 Interesting functionalities of a digital solution for GHD management

Tailoring the content of the digital health solution was considered an interesting feature to support GHD management. Although both panels agreed on most of the important factors, the Italian panel emphasised the sociocultural level of patients/caregivers, whereas the Korean panel highlighted the character of the individual.

> *"We think a lot about the hardware factor but also in terms of the software, we should think about what the contents of the reminder would be and even if the contents are the same, what the nuance is going to be and how to make it more encouraging, whether emojis would be used, etc. All these would be very important factors to consider. Also, we should decide whether it would target the children or the caregivers, and tailor for each patient group." [Korea]*
> *"Instead, for the rest, it is a last point. I would put it's easy. Transmission, data reliability, ability to maintain data over time. So, these are the three most important things. Cultural, socio-cultural reasons" [Italy]*

Both panels agreed to offer support in the management of GHD through digital health solutions, with the Korean panel contributing to some technical aspects, such as the use of video or artificial intelligence tools (chatbots). They also highlighted the desire to provide just-in-time support.

> *"So that's why I think a chatbot function would be useful because the patients can ask questions to it or ask what to do in certain situations. I used to think that all these questions in a chatbot were answered by human but later, I found out that it wasn't. So, if such algorithm is developed, this will lessen the workload of HCPs and also enable caregivers to obtain accurate information at the same time." [Korea]*

Reminders and motivational messages were considered relevant functionalities by both panels; however, the Korean panel discussed more technical details such as frequency or content.

> *"While not many actually do as told, I think it still helps them to take their medications regularly. So such alarm or a tool that allows us to check (compliance) would be ideal." [Korea]*
> "Interestingly, if the patient does not take the dose for several days, he sends an alert" [Italy]

Both Korean and Italian experts agreed that providing feedback to patients/caregivers was a relevant functionality. In this regard, both panels identified several technical aspects, with the Korean panel highlighting some advanced features, such as the inclusion of gamification elements. Additionally, this panel pointed out rewards schemes and discussed this topic at length.

> *"We say positive feedback. Rewards and positive feedback. The patient sees the smiley faces, he feels good. Quality of life, he is happy" [Italy]*

3.3 Perceived benefits and risks of using digital health solutions for adherence monitoring

3.3.1 Healthcare system level

From the point of view of the healthcare system, a few differences were found between the two countries. Although both Korean and Italian panels considered resource optimisation and cost reduction as potential benefits of automatic adherence data collection, the Italian panel perceived that these data could lead to fewer laboratory tests and less drug wastage, whereas the Korean panel focussed on financing issues, especially for insurance-financed treatments.

> *"Healthcare system. Less drug waste, therefore economic impact. Who cares then, the only thing, then they would be able to put in more visits if the visits last less, because we are in bad shape. Use number and times because you make fewer visits" [Italy]*

> *"Regarding insurance,…, in the case of continuous glucose monitoring, the policy is that the government won't cover the cost for those with a compliance level of under a certain level. So also with GHD, if the adherence is a lot less than expected from accumulated data, and yet a lot of patients are receiving reimbursement, this might enable changes in reimbursement paradigm." [Korea]*

The Italian panel felt that automatic adherence data collection would allow them to optimise motivational strategies to encourage patients/caregivers to comply with GHD treatment. Additionally, the panel identified the potential risk of using these data when measurements are not accurate (e.g., dose detection and fake injections).

> *"From a therapeutic point of view, I see the consequences only in a positive sense. Obviously, the therapy, maximized clinical efficacy, that's right. The middle ground: patient support is needed mostly from the HCP and from the national health service. The middle ground is the crisis between the two. Malaise for mistakes, possible? It's like that, in the sense, it's not accessible to everyone, because it's an application. Sharing, how can the patient perceive it? As a task to which he has to obey, let's say, an added task, a boredom of having to mark things down? A further burden is the last thing, the fact that if the data is not correct there is a risk." [Italy]*

3.3.2 HCP level

In terms of potential benefits in decision-making, both Italian and Korean HCPs reported that having automatically collected data would allow them to apply more personalised and just-in-time interventions.

> *"In this system, would there be a way for us to see patients with bad adherence and give feedback? Usually, those who are good are not the ones that we have to worry about, and the purpose of this (system) is to find those who are in need of help. So, if we could find ways to provide feedback to encourage those with bad adherence, such as providing happy calls, it might be helpful" [Korea]*

The Italian panel perceived some potential inequities because of the lack of digital skills of HCPs, lack of training of HCPs, or lack of non-digital alternatives. Both panels agreed that checking the collected data could lead to increased workload. The Korean panel had a more negative perception, with some of the experts reporting that HCPs could feel guilty for not being able to check collected data as expected by patients/caregivers. Although the Italian panel agreed, they felt that HCPs may even feel more confident because they could use more accurate and reliable data to optimise patient treatments.

> *"However, regarding the third question of whether HCPs will welcome using this data, we are already experiencing a high workload and it will immediately be recognised as a burden. So, I think there should be more reward for HCPs than just being able to better take care of their patients. For example, there is a code for a medical fee for provision of CGMs. Likewise, if they could provide a separate code for growth assessment of children receiving GHD treatment, that could be a reward for us for monitoring these patients more closely and going the extra mile for analysis" [Korea]*
>
> *"Because we usually see GHD patients quite frequently for more than 5 years, GHD patients are one of the patient groups that we usually have good rapport with. So, I don't think we've had much issues in terms of sharing data and on the contrary, I think I often felt bad about not giving them enough feedback on the data provided" [Korea]*
>
> *"This here data accuracy, reliable, absolutely certain, objective" [Italy]*

3.3.3 Patient/caregiver level

Both Korean and Italian experts agreed that the use of these digital health solutions helps in prescribing evidence-based therapies that could lead to patient/caregiver empowerment. They linked tracking adherence data to high levels of empowerment.

> *"Scenario 3 (the use of Aluetta® with Smartdot™ and the connected digital ecosystem to gather adherence data) the patient's empowerment and self-efficacy improve markedly. Minimum effort, because the patient has to make a minimum of effort to improve the results. Empowerment when the therapy is followed correctly and the fact that the doctor interacts in real time remotely." [Italy]*
>
> *"I agree. Of course, there will be a lot more ways and items to be able to link and observe growth curve on the app, which will be beneficial in terms of patient empowerment" [Korea]*

Both Korean and Italian experts believed that automated adherence data collection could influence patient/caregiver motivation and promote adherence. Korean experts agreed that the feedback features would impact the ability of patients/caregivers to self-monitor their GHD and, therefore, improve their self-management. Similarly, Italian experts agreed that the use of digital health solutions can facilitate timely feedback. Some of the experts reported that digital solutions can maximise the opportunities to provide feedback to patients and caregivers. These experts linked feedback to high levels of motivation and satisfaction among children and their families.

> *"We have written about the importance of effectiveness in terms of growth, which returns a fairly evident result that brings satisfaction to both the family and the child himself" [Italy]*

> *"So, in clinic, regarding health management from the caregiver's perspective, I think it could give them an impression that their physician is paying attention and caring for them" [Korea]*

Regarding the potential risks associated with the use of digital health solutions, the Italian panel reported that a lack of digital literacy could lead to health inequities. However, the Korean panel considered that a non-digital alternative should be implemented, allowing patients/caregivers to choose their preferred option. Additionally, some of the Korean experts commented on the possibility of patients feeling controlled and potential data privacy issues causing reluctance in data sharing.

> *"But even so, patients may prefer the notebook, because using the app would mean disclosing a lot of their private information, which may act as a resistance factor. Writing the values down in a notebook might feel like they are keeping a secret to themselves, while using an app automatically means that the data is accessible by others, which I think will be associated with resistance. So, we need to take into consideration the privacy issue and there should be a way to protect that" [Korea]*

3.3.4 HCP–patient relationship

All experts agreed that automated adherence data collection provides them with accurate and reliable data that they can use to communicate with their patients/caregivers. Automated collection could increase trust between HCPs and patients/caregivers. However, the Korean panel reported that more reliable and accurate data could lead to conflicts between patients and caregivers, which would negatively impact the HCP–patient relationship. They reported that some patients may also be reluctant to come for further visits if HCPs determined that they were lying.

> *"As repeatedly mentioned, from management perspective, availability of objective data allows us to build further trust with caregivers. When such tool was not available, I had a diabetes patient whose adherence was really bad despite constantly being told that she needs to improve. So, I gave her an ultimatum by saying that I'd have to transfer her to a different clinic, and she started crying and said that I never encouraged her by saying that she had also been good" [Korea]*

The Italian panel provided mixed opinions. Some experts commented that these objective data could provoke negative feelings of control or intrusiveness among some patients. Conversely, others felt that the HCP–patient relationship may improve because patients/caregivers may feel that HCPs were taking care of them.

> *"There are two points: trust and improvement of the doctor-patient relationship and surveillance" [Italy]*

3.4 Perceived usefulness and ease of use of Aluetta® with Smartdot™ and the Growzen™ ecosystem

Experts from both countries agreed that Aluetta® with Smartdot™ had a user-friendly format for transforming a pen into a digital health solution. They did not perceive any changes in terms of weight when the Smartdot™ accessory was attached to the Aluetta® pen, making it suitable for use by children.

> *"Considering the size of the pen, which is substantial, I think they've done their best with the technologies available to minimise the cap size" [Korea]*

Although some Korean experts commented on the desirability of incorporating some form of feedback to indicate that the coupling process has been successfully completed, most of the Italian experts reported that they missed receiving feedback and preferred to receive audio feedback.

> *"It would take something like click, which gives the feeling that it engages, in my opinion. It would take a shot when you put it" [Italy]*

Between the two countries, the main difference was highlighted in charging. The Italian panel expressed concerns about battery life, fearing that forgetting to charge the device could result in data loss. Conversely, the Korean panel emphasised the technical aspects of charging, expecting a more advanced process like wireless charging to make it easier and prevent data loss.

> *"Lastly, it should be easy to charge the device, for example, adopting a wireless charging system that will automatically charge the device once it's placed and stored in the case, rather than having to charge every two weeks. So, these are the five suggestions" [Korea]*

Apart from these features, the HCPs from both countries also shared their comments on the perceived ease of use of Aluetta® with Smartdot™, as summarised in Table 1, and the Growzen™ digital health ecosystem. The Growzen™ Buddy patient app was considered as an easy-to-use application by experts from both countries.

> *"The application, what do we think? It is objectively easy; you immediately understand how to use it;" [Italy]*
> *"Regarding how to attract the existing Aluetta® users, one of the biggest barriers will be how fast they get used to the application. In other words, we have to minimise time wasting that may arise from the app" [Korea]*

TABLE 1 Summary of experts' comments on the perceived usefulness and ease of use of Aluetta® with Smartdot™.

Component	Topic	Summary of comments from Italy	Summary of comments from Korea
Aluetta® with Smartdot™	Location of components	The administration button of Aluetta® with Smartdot™ was easier to use and more ergonomic, improving the user experience.	The device was manageable and its dimensions made it suitable for use by both adults and children.
	Pairing and configuration	Aluetta® with Smartdot™ configuration in the Growzen™ Buddy patient app was easier to do than the new smartphone configuration.	The pairing process was found to be similar to that used in other current Bluetooth® devices.
	Ease of use	Aluetta® with Smartdot™ was quite similar to other electronic devices, and people who were familiar with it could easily use it. Aluetta® with Smartdot™ improved the usability of the pen.	Aluetta® with Smartdot™ mounted was lightweight and perfectly suitable for children.
	Reliability and accuracy	–	Aluetta® with Smartdot™ may be helpful in analysing the cause of non-adherence and objectively verify patients with poor adherence by documentation.
	Target users	–	Aluetta® with Smartdot™ will likely be used by new patients and existing adherent patients.
	Perceived risks		Aluetta® with Smartdot™ may have issues with connection due to refrigeration.

The use of the Growzen™ Buddy patient app was perceived to be easy for healthcare professionals to teach and for patients/caregivers to learn.

> *"It is easy to explain to an assistant/patient how to configure and associate the device with the APP" [Italy]*
> *"Is the setup easy to learn? It's easy, also because you don't need to update" [Italy]*
> *"I think such app would really help. If we provide thorough explanation at first, it should be useful for patients and caregivers" [Korea]*

Regarding appearance, both Italian and Korean experts found the graphical user interface of the application to be user-friendly and appropriate for patients/caregivers.

> *"The colours, the icons, the combination of data; navigation is easy and intuitive, is it clear and understandable? I would say that these 5 are perhaps the most interesting. Even the reminder isn't bad, but there's not much to say about the reminder. Does the mobile app help users gain more insight into their adherence behaviour? Yes, of course, that's why it's made, so I'd say it's the most fitting" [Italy]*
> *"So maybe the users will be interested in the initial phase, because it kind of looks like a game, but in order to maintain the momentum, then we would need to provide some kind of reward. And while it may depend on the character of the patients, those who show good growth would probably have a high satisfaction level with the application" [Korea]*

Experts from both the Korean and Italian groups commented that the Growzen™ Buddy patient app implemented several feedback strategies that would positively impact patients in their adherence.

> *"Is feedback useful when it has been configured? Very helpful" [Italy]*
> *"The mobile app helps users to get more information about their joining behaviour. The most important of all" [Italy]*
> *"In a sense that this device is about recording the treatment history, I think it could be meaningful for them to see the record of treatment history, And I think this will be positive impact on caregivers" [Korea]*

Italian experts agreed that the Growzen™ Connect HCP platform can be used to engage and discuss reports with patients/caregivers.

> *"The platform is our stuff. APP is something mobile, SMS is mobile, we can put the platform just www.growzenconnect.com. We put it on reports, it is consulted only by the healthcare provider, therefore only by us. But it is something that obviously serves the patient's purposes, therefore something digital in any case." [Italy]*

4 Discussion

This study comprehensively explored HCPs' perspectives on the adoption of digital health solutions and the acceptance of a digital device ecosystem across Korea and Italy. Understanding the nuances of these perspectives is indispensable for developing strategies to overcome the challenges and leverage the opportunities presented by the ongoing digital transformation in healthcare. Although HCPs appreciate the potential of digital health solutions to improve patient engagement and, hence, clinical outcomes, the participatory workshops revealed several aspects on how this digital transformation is impacting treatment options and the need for digital literacy for successful implementation (Figure 3).

The method employed to conduct the participatory workshops facilitated the collection of perspectives of experts from universities and hospitals across Korea and Italy, wherein they shared clinically valuable and understandable technology acceptancy information governing potential barriers and facilitators for the use of Aluetta® with Smartdot™ and the Growzen™ Buddy patient app and the Growzen™ Connect HCP platform. The qualitative analysis compared the opinions of the experts considering two different groups—Italy and Korea—and a researcher reviewed all themes and comments included in them. It was observed that the healthcare systems in both countries are different. As digitalisation was well accepted in Korea given the technical readiness and awareness among patients, HCPs' adaptation to digital health solutions could be positive. Conversely, the Italian national health system had limited human and technical healthcare resources to support the GH digital health ecosystem, and patients'/caregivers' adoption of digital health solutions varied depending on an individual's characteristics, skills, or motivation level. With the spectrum of options available with patients and caregivers, the choice of digital health solution may impact adherence. Therefore, it would be crucial to consider the specific needs and preferences of the patients, caregivers, and HCPs and have features that could be useful to support patients/caregivers in managing their conditions in both countries. The analysis revealed some of the risks and benefits associated with the use of digital solutions for adherence monitoring, such as accessibility to adherence data, data-driven clinical decisions, visibility of results, and strengthened HCP–patient relationships. HCPs from both countries perceived Aluetta® with Smartdot™ as an excellent digital health solution for GH therapy that can create scientific evidence on the relationship between adherence and efficacy.

Over many years, the digital devices for diagnosis, treatment administration, and monitoring have evolved with technological advances in mobile connected health, artificial intelligence, digital patient support programmes, telemedicine, and gamification using virtual and augmented reality (3). To date, the perspectives of HCPs towards digital health solutions have not been studied for paediatric GH therapies using injector pens. This article presents first-of-its-kind insights that emphasise the benefits of the digital ecosystem, constraints of HCPs and the need to address important aspects related to the acceptance of such technology upgrades. Many of the statements from the clinicians reinforced the adherence support described by the World Health Organization, which included elements such as literacy and support (17). Multiple clinicians highlighted the importance of having

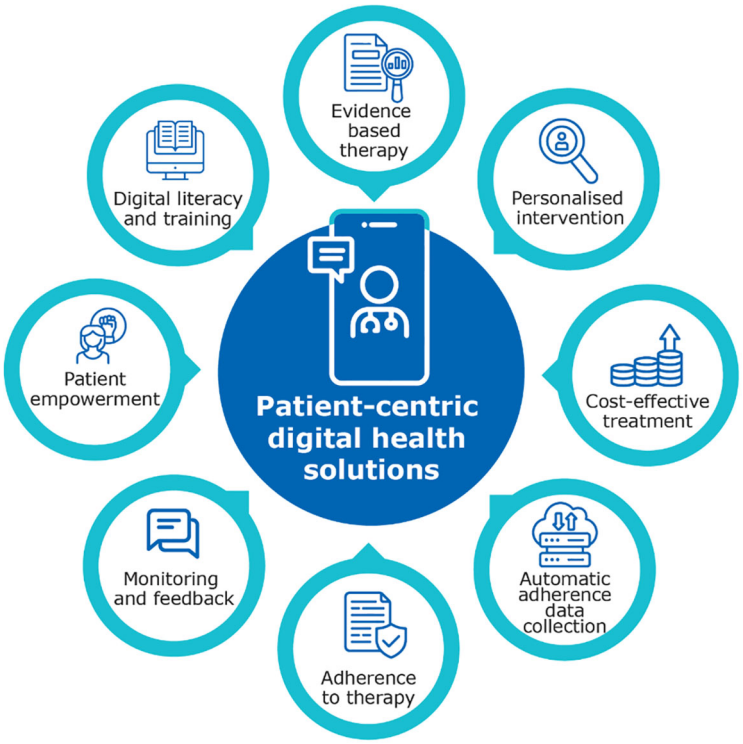

FIGURE 3
Patient-centric digital health solutions.

more adherence data to improve clinical practice and research. This feedback is congruent with recent reviews on the use of sensors to monitor adherence (18, 19).

However, the use of connected sensors for adherence can affect the cost effectiveness of the treatment, which is not always quantified and recognised by healthcare systems. The importance of the value of adherence data appears to be clearly linked to the provision of visualisations to facilitate condition management, which also includes the visualisation of data in both the mobile application and interface for the doctors. There are emerging initiatives on creating standards for adherence reporting that also mention such needs; however, more research is required in that area (20). In the case of connected injection pens, it is essential to consider that the user interfaces encompass not only the connected pen but also the mobile application used for pairing process to link the Aluetta® with Smartdot™ with the Growzen™ Buddy patient app.

All HCPs highlighted that a connected injector device such as Aluetta® with Smartdot™ in the Growzen™ ecosystem can help personalise care by enabling patient empowerment and clinical decision-making. Aluetta® with Smartdot™ was considered to be easy to use, easy to learn and teach, ergonomically suitable for use by both children and adults, comfortable to be transported, robust, easy to charge, and easy to pair with other devices, thereby providing a better administration experience. From HCPs perspective, the Growzen™ Buddy patient app would be easy to use, easy to learn, and the feedback provided by the application would be valuable to motivate patients. Growzen™ Connect HCP platform was considered useful for data analysis by HCPs and for promoting discussion with patients/caregivers. Furthermore, the following aspects were considered actionable: 1) healthcare systems need to include adherence monitoring as part of pharmacoeconomic models considered by payors; 2) training on the use of adherence data derived from connected devices should be promoted to both clinicians and patients; 3) easy-to-use platforms that support HCPs in data analysis should be accessible, including alerts when events requiring attention occur, such as actionable recommendations when a lack of adherence is detected or predicted; 4) the possibility of prediction tools based on newly captured data should be explored to bring about a positive impact on research; 5) digital literacy and privacy concerns experienced by some users should be addressed, and the potential negative impact of using digital health or health disparities should be reduced; and 6) best practices to incorporate such sources of data into the provision of care should be studied, especially considering the impact on clinicians' time.

One of the challenges observed in the true adoption of a digital health ecosystem is the long-term engagement of HCPs and patients/caregivers with digital health applications/devices. Often owing to the limitations of time or digital literacy, sustained engagement with technology poses a challenge. Sensor-based devices such as Aluetta® with Smartdot™ present with an alternate communication platform that is essential to engage patients/caregivers and develop user-centered solutions for the treatment and management of GHD (21).

The participatory workshops in these two countries examined the perspectives of a small group of experts over a short period. Further studies are required to determine the extent of digital health solution adoption among HCPs and patients/caregivers. Furthermore, with the progression and evolvement of technology, some desired features discussed may be incorporated, and HCPs' recommendations may be altered. Although these perspectives may not be universal, they do help in the development of an individualised approach to GH treatment.

5 Conclusion

HCPs are one of the foremost stakeholders in the implementation of digital health solutions. Our participatory workshop helped capture meaningful insights from them as experts. The main findings highlighted that experts considered/perceived Aluetta® with Smartdot™ within the Growzen™ digital health ecosystem as user-friendly, intuitive, and easy-to-use digital health solutions. Aluetta® with Smartdot™ enabled automatic, real-time injection data transmission to support adherence monitoring and data-driven treatment decisions, thereby helping understand the reasons for suboptimal response or adherence issues with GHD therapy. The availability of unbiased, reliable, and accurate data transmitted by the device would be beneficial and help generate new evidence-based knowledge to support GHD therapy, strengthen patient–HCP relationships, and empower patients throughout the treatment process. The findings from these workshops can further contribute towards novel insights to enable HCPs to better adopt and prescribe digital health solutions as part of their routine care and support researchers with new clinically relevant datasets for better management of GHD.

Data availability statement

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding authors.

Ethics statement

All procedures performed in this study were in accordance with European and national ethical guidelines, the European Code of Conduct for Integrity in Research, the Universal Declaration of Human Rights and the Helsinki declaration. Each participant was informed about the research topic and procedures before joining the expert panel. They signed an agreement giving their consent to participate. Participants in this expert panel were not considered human subjects. The main topic of this research was the digitization of healthcare. The experts provided their opinions, based on their experience, on this topic by assuming an advisory role. Therefore, they were not the main subjects of the study. In this study, the experts' opinions included as quotes in the paper were pseudo- anonymised. No sensitive information was used or collected, and the contributions of the expert panel had no impact on others. In addition, no interventions were made in this study. Therefore, no additional ethical approval was deemed necessary. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

ORR: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. HWC: Conceptualization, Methodology, Validation, Writing – review & editing. MFF: Conceptualization, Methodology, Validation, Writing – review & editing. EV: Conceptualization, Methodology, Validation, Writing – review & editing. CKC: Conceptualization, Methodology, Validation, Writing – review & editing. RDM: Validation, Writing – review & editing, Conceptualization, Methodology. FF: Conceptualization, Methodology, Validation, Writing – review & editing. HSL: Conceptualization, Methodology, Validation, Writing – review & editing. CG: Conceptualization, Methodology, Validation, Writing – review & editing. JK: Conceptualization, Methodology, Validation, Writing – review & editing. AK: Conceptualization, Methodology, Validation, Writing – review & editing. JEM: Conceptualization, Methodology, Validation, Writing – review & editing. MLI: Conceptualization, Methodology, Validation, Writing – review & editing. JY: Conceptualization, Methodology, Validation, Writing – review & editing. AA: Conceptualization, Methodology, Validation, Writing – review & editing. Y-JR: Conceptualization, Methodology, Validation, Writing – review & editing. EK: Conceptualization, Methodology, Validation, Writing – review & editing.

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Conflict of interest

All attendees (HCPs) received a fee for participation. EV received fee as consultant from Merck Serono Italy. ORR has participated in an advisory board for Merck. EK is an employee of Merck Healthcare KGaA, Darmstadt, Germany, and holds shares in the company. JY is an employee of Merck Ltd., Seoul, South Korea, an affiliate of Merck KGaA, Darmstadt, Germany.

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Meta-analysis and systematic review: burosumab as a promising treatment for children with X-linked hypophosphatemia

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Objective: The aim of this study was to evaluate the effectiveness of burosumab therapy in children with X-Linked Hypophosphatemia (XLH).

Materials and methods: We systematically reviewed literature from PubMed, Web of Science, The Cochrane Library, and Embase up until January 2024, using EndNote Web for study organization. The Newcastle–Ottawa scale guided quality assessment, while Revman software was used for data analysis and visualization. Study selection, quality evaluation, and data aggregation were independently performed by three researchers.

Results: The meta-analysis encompassed ten studies, including eight cohort studies that examined burosumab's impact pre- and post-administration, and two randomized controlled trials comparing burosumab to standard therapy. The evidence from this review suggests burosumab's superiority in managing XLH in pediatric populations, particularly in improving key biochemical markers including 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D), phosphorus, and alkaline phosphatase (ALP), alongside improvements in the renal tubular maximum reabsorption rate of phosphate to glomerular filtration rate (TmP/GFR), and significant skeletal improvements as indicated by the rickets severity score (RSS) and the 6-minute walk test (6MWT). However, the long-term safety and effects, including height and quality of life (QOL) data, remains to be elucidated.

Conclusions: Burosumab has shown significant therapeutic effectiveness in treating children with XLH, highlighting its potential as a key treatment option.

KEYWORDS

X-linked hypophosphatemia, burosumab, pediatric, meta-analysis, therapeutic effectiveness

1 Introduction

X-linked hypophosphatemia (XLH) is a rare genetic disorder primarily caused by loss-of-function variants of phosphate-regulating endopeptidase gene on the X chromosome (PHEX) located on the X chromosome. It is the most common form of hereditary rickets and osteomalacia (1, 2), while other genetic factors can also contribute to hypophosphatemic rickets, including mutations in FGF23, DMP1, ENPP1, and FAM20C (3). With a prevalence of approximately 1 in 20,000 newborns, XLH presents significant clinical challenges (4, 5). The disorder's primary manifestations in infancy include rickets and growth retardation, which evolve into more pronounced lower limb deformities during childhood and adolescence (6). Additionally, studies indicate that XLH children also suffer from bone pain, gait impairment, reduced range of motion of ankle/knee joint, tooth abscesses, and/or skull stenosis (6–8).

XLH is characterized by reduced renal phosphate reabsorption, which can be reflected by TmP/GFR value (9, 10), and impaired production of 1,25-dihydroxyvitamin D ($1,25\text{-}(OH)_2D$), complicating its management (11–13). The conventional therapy of XLH is treating the patients with oral phosphate and active vitamin D to compensate for renal phosphate wasting and counter $1,25\text{-}(OH)_2D$ deficiency (14). With traditional treatment methods, the serum alkaline phosphatase (ALP) level can be successfully regulated to remain within the upper limit of normal values within a year (14). Additionally, this treatment has shown efficacy in improving bone deformities in 30% to 60% of patients, enhancing their growth velocity, and optimizing dentin mineralization (14–16). While this approach can modestly improve bone deformities and growth rates in pediatric patients, its efficacy varies, and long-term use is associated with significant adverse events, including hyperparathyroidism, hypercalciuria, renal calcinosis, and kidney stones (14, 17). Despite treatment, hypophosphatemia often remains unresolved, and full restoration of muscle function is not achieved. Due to insufficient response to medication, some children require corrective surgery on their lower limbs (18). Furthermore, the frequent dosing required for conventional treatment poses a significant hurdle in ensuring patient compliance, as phosphate must be taken several times a day. These challenges underscore the urgent need for more effective and safer treatment options to enhance the quality of life for children with XLH.

In exploring alternative treatments, recent research has focused on the role of Fibroblast Growth Factor 23 (FGF23) in XLH pathophysiology. FGF23 are significantly elevated in XLH patients, which is responsible for renal phosphate wasting and suppressed $1,25\text{-}(OH)_2D$ expression (19–21). Produced mainly by osteocytes and osteoblasts, FGF23 plays a crucial role in phosphate homeostasis. Its function on phosphate metabolism was initially recognized by missense variants in FGF23 discovered from children with autosomal dominant hypophosphataemic rickets (ADHR) (22). The variants found in ADHR patients prevent the normal cleavage of the intact, bioactive form of FGF23, leading to low serum phosphate concentrations and rickets/osteomalacia (11, 23). FGF23 regulates the reabsorption of phosphorus by inhibiting the expression of the sodium phosphate co-transporters, NPT2a and NPT2c, at the renal proximal tubules (24). It also represses the synthesis of active $1,25\text{-}(OH)_2D$ and enhances its degradation by upregulating the renal 24-hydroxylase enzyme (CYP24A1) and downregulating the 1α-hydroxylase enzyme (CYP27B1) (25, 26). Moreover, FGF23 impacts parathyroid hormone (PTH) expression, influencing blood calcium and phosphate metabolism and ultimately affecting bone mineralization, leading to compensatory changes in osteoblast activity and alkaline phosphatase secretion (27).

Burosumab, a monoclonal antibody targeting FGF23, has emerged as a promising new treatment. Approved by the European Medicines Agency, the U.S. Food and Drug Administration, and other regulatory bodies for both pediatric and adult XLH patients, burosumab works by blocking FGF23, thereby increasing renal phosphate reabsorption and enhancing serum levels of phosphorus and active vitamin D. Clinical trials have demonstrated that burosumab significantly improves serum phosphate levels, increases active vitamin D levels, and enhances renal phosphate reabsorption. Additionally, the therapeutic effects of burosumab extend beyond biochemical improvements. QOL assessments have shown marked enhancements in patients with burosumab treatment, with reports of reduced pain, increased physical activity, and overall better well-being (28, 29). There is evidence of sexual dimorphism in XLH severity, with males often exhibiting more severe symptoms than females (5, 30). Burosumab's effectiveness appears consistent across genders, though further studies are needed to confirm this (31, 32). Furthermore, burosumab has a favorable safety profile with fewer adverse events compared to conventional therapy, making it a more viable long-term treatment option (33). These benefits underscore burosumab's potential to offer a more comprehensive and effective treatment solution for XLH, addressing both the physiological and QOL aspects of the disorder. However, the specific impact of burosumab on children with XLH warrants careful evaluation through clinical research.

This article aims to conduct a comprehensive meta-analysis and systematic review of the available clinical trials on burosumab's use in treating pediatric XLH. We intend to scrutinize multiple study data, critically evaluate the advantages and limitations of burosumab, and offer a reliable assessment for future drug research and development directions. Our goal is to contribute to the growing body of knowledge on XLH treatment and to provide insights that may guide clinical practice and improve patient outcomes.

2 Materials and methods

2.1 Protocol and registration

This study was conducted and reported in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist (34). It has been registered in the Prospero database with the registration ID CRD42023424461.

2.2 Eligibility criteria

Articles evaluating the therapeutic impact of burosumab in pediatric XLH patients, in comparison to other treatment modalities, were considered. There were no restrictions on

publication date or language. The inclusion criteria were structured according to the PICOS question as follows:

Population (P): Pediatric individuals diagnosed with XLH.

Intervention (I): Burosumab treatment.

Comparison (C): Other treatment approaches.

Outcome (O): Rickets severity and related parameters.

Study design (S): Cross-sectional, randomized-control, and cohort studies examining serum parameters, rickets development, or walking ability among participants.

Exclusion criteria encompassed studies without a control group of pediatric XLH patients not receiving burosumab, those not reporting relevant serum parameters, rickets development, or walking ability, case reports or series, literature reviews, studies lacking statistical analysis, and qualitative studies. Studies focusing on outcomes other than rickets were also excluded.

2.3 Information sources

Our search encompassed four electronic databases: PubMed (https://www.pubmed.gov), Web of Science (https://www.isiknowledge.com), The Cochrane Library (https://www.cochranelibrary.com), and Embase (https://www.embase.com) from their inception up to January 2024. The list of identified studies was organized using EndNote X9, and duplicate records were removed.

2.4 Search strategy

The search strategy included terms: ((Burosumab or KRN23 or Crysvita) AND (X-linked hypophosphataemia or XLH or hypophosphataemic rickets) AND (pediatric OR children)).

2.5 Study selection

An initial pool of 667 articles was identified, which was subsequently narrowed down to ten publications for inclusion in the meta-analysis. This selection process, conducted by reviewers Kangning Wang, Runze Zhang, and Ziyi Chen, involved three stages: first, the use of EndNote X9 to identify and eliminate duplicate records; second, an individual review of titles and abstracts for relevance; and third, a full-text analysis to finalize selections. Discrepancies were resolved through consensus.

2.6 Data collection process and data items

Three reviewers (Kangning Wang, Runze Zhang, and Ziyi Chen) independently extracted data from the selected articles, including study design, timeframe, follow-up period, participant demographics (country, setting, age, sex distribution), interventions, and outcomes (serum phosphorus levels, serum 1,25-$(OH)_2$D levels, ALP levels, TmP/GFR, rickets severity score (RSS), Height Z-Score, 6-minute walking test results (6MWT), etc.). Authors of the studies were contacted for additional information when necessary.

2.7 Risk of bias in individual studies

The risk of bias was independently evaluated by the three reviewers (Kangning Wang, Runze Zhang, and Ziyi Chen) using the Newcastle–Ottawa scale for randomized-control and cross-sectional studies. Each item in the selection and exposure groups was eligible for a maximum score of one point, while each item in the comparability group could receive a maximum of two points. The highest possible score was nine. Studies were scored and categorized into high (7–9), medium (4–6), or low quality (below 3).

2.8 Data synthesis and statistical analysis

Estimation of aggregate effect size and forest plot generation were performed with the RevMan 5.4 software. In experiments with a before-and-after control of the cohort studies, a cohort analysis was performed, and randomized controlled trials were analyzed using randomized-control study methodology. The confidence interval (CI) for the included studies in the forest plot was set at 95%. The standardized mean differences (SMDs) with the 95% CIs of each parameter were calculated, and the significance threshold was set at $P < 0.05$. Subgroup analysis evaluated the effects between burosumab treatment and conventional treatment, calculating combined effect size (ES) and variance, and displayed using forest plots. The I^2 (percentage of variability in the effect sizes), tau-squared (between-study variance), and Cochran's Q test (difference between the observed effect sizes and the fixed-effect model estimate of the effect size) statistics were tested for statistical heterogeneity.

3 Results

3.1 Study selection

Our electronic searches yielded 667 titles and abstracts. In the initial screening phase, 383 titles and abstracts were reviewed, with 284 excluded due to duplication. Subsequently, 74 studies underwent full-text analysis. Reasons for exclusion at this stage included experimental duplication (36 articles), irrelevance to the topic (2 articles), reviews (4), and insufficient outcome indications (21 documents). Of the 11 studies initially included in qualitative synthesis, one was excluded due to an unbalanced gender ratio at the selected time point. Consequently, ten publications were included in this systematic review and meta-analysis. Six of these compared burosumab with conventional therapy in children (31, 32, 35–38), while the other four assessed burosumab's efficacy and safety versus control (39–42). A thorough manual search did not yield additional articles. The article selection process is visually depicted in Figure 1.

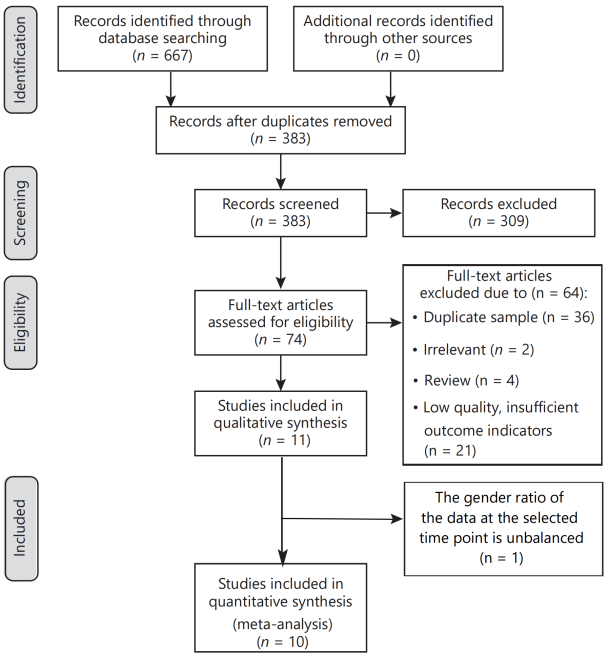

FIGURE 1
Flow diagram of the systematic review and meta-analysis.

3.2 Characteristics of the included articles

Table 1 summarizes key characteristics of the included studies, covering ten distinct experiments on burosumab's effectiveness in XLH patients. One study examined the efficacy of burosumab administered biweekly (Q2W) versus every four weeks (Q4W), transitioning to Q2W after 64 weeks (41). One study was controlled trials comparing burosumab with an active control group (32). Two trials explored the effects of biweekly burosumab injections over periods ranging from 114.8 weeks to 160 weeks, involving participants aged 2.94 ± 1.146 to 7.40 ± 3.40 years on average (39, 42). Six studies investigated the transition from conventional therapy to Q2W burosumab injections (31, 35–38, 40), with one study noting that 94% of participants had previously received conventional therapy, and this trail was not included in subgroup analysis (40). The studies adopted the age of twelve as the demarcation point to differentiate between children and adolescents. Six studies exclusively included children (32, 37–39, 41, 42), while four studies included both children and adolescents (31, 35, 36, 40). In one of these studies, treatment outcomes for children and adolescents were documented separately, and for our analysis, we solely incorporated the results pertaining to children (31). Sample sizes across these studies varied from 5 to 93 participants, with diverse injection dosages, cycles, and experimental durations. At the final time point chosen (40 weeks), among the studies that measured TmP/GFR, six studies included the TmP/GFR of all patients (31, 36–40). Due to the difficulty of collecting urine samples from young children, the studies by Imel et al. and Linglart et al. did not measure the TmP/GFR of all participants (32, 41). Specifically, the study by Imel et al. omitted 10 participants (6 from the burosumab group and 4 from the control group), and the study by Linglart et al. omitted 3 participants (2 from the burosumab group and 1 from the control group).

3.3 Risk of bias in individual studies

The Newcastle–Ottawa scale was applied to determine included study quality (Tables 2, 3). Ten included studies were categorized as either randomized-control or cohort studies, and different evaluation forms were employed to assess them accordingly. The eight cohort studies scored 7-8 points, denoting high quality (31, 35–37, 39–42) (Table 2), while the two randomized-control studies achieved full marks, designating them as high-quality articles (32, 38) (Table 3).

3.4 Meta-analysis for the effects of burosumab

For the meta-analysis of burosumab treatment outcomes, forest plots were employed to comprehensively assess overall effects, including 1,25-$(OH)_2$D, serum phosphorus, TmP/GFR, RSS, ALP, 6MWT, and Height Z score.

3.4.1 1,25-$(OH)_2$D

Seven studies with a total of 400 patients were included in the analysis of changes in 1,25-$(OH)_2$D before and after therapy (31, 32, 37, 39–42) (Figure 2). A significantly greater increase in 1,25-$(OH)_2$D levels in the burosumab group compared to the control group (SMD 21.73, 95% CI 12.43-31.03, P <0.00001), albeit with

considerable heterogeneity (I^2 = 84%). Sensitivity analysis pinpointed two studies, Kubota, T. et al. and Namba, N. et al. (37, 39) as major heterogeneity sources due to their low baseline values. Excluding these studies did not alter the overall conclusion, reinforcing burosumab's effectiveness in addressing vitamin D metabolism in pediatric XLH patients (Figure 3).

3.4.2 Serum phosphorus

This outcome was analyzed using data from ten studies (31, 32, 35–42) (Figure 4). The analysis of these ten publications collectively demonstrated a notable increase in serum phosphorus levels in the burosumab group compared to controls (SMD 0.9, 95% CI 0.82-0.99, P<0.0001), with moderate heterogeneity (I^2 = 47%). The analysis robustly demonstrates burosumab's role in correcting hypophosphatemia in XLH children.

3.4.3 TmP/GFR

To evaluate changes in TmP/GFR before and after therapy, data from eight studies were used in this analysis (31, 32, 36–41) (Figure 5). The analysis demonstrated a significant increase in TmP/GFR in the burosumab group (SMD 1.22, 95% CI 1.02-1.43, P<0.00001). The I^2 statistic was 83%, showing a significant heterogeneity. Sensitivity analysis showed that the heterogeneity came from Ewert, A. et al. study (31), which had a lower Mean Difference (MD) (Figure 6). Exclusion of this study leaded to consistent conclusion. The significant improvement in TmP/GFR ratios with burosumab treatment, even after adjusting for heterogeneity, underscores its effectiveness in enhancing renal phosphate reabsorption.

3.4.4 RSS

Six studies contributed to RSS analysis (32, 37, 38, 40–42) (Figure 7). Burosumab group showed a significant reduction in RSS compared to controls (95% CI 1.41-1.27, P<0.00001), without significant heterogeneity (I^2 = 0%). The reduction in RSS scores in the burosumab group strongly suggests that burosumab is effective in improving radiographic outcomes for XLH patients.

3.4.5 ALP

Eight studies were analyzed for ALP parameter, indicating a significant reduction in ALP levels in the burosumab group (31, 32, 35, 37, 38, 40–42) (Figure 8) (SMD -125.98, 95% CI −152.79-−99.17, P<0.00001). The I^2 statistic was 62%, showing a significant heterogeneity. Sensitivity analysis showed that the heterogeneity came from Namba, N. et al. (37). study, which exhibited a significantly higher ALP value compared to other studies (Figure 9), and heterogeneity was resolved by excluding this study. The substantial decrease in ALP levels in patients treated with burosumab indicates its efficacy in normalizing bone turnover markers in XLH.

3.4.6 6MWT

The assessment of changes in the 6MWT included three studies (32, 37, 41), showing the burosumab group's 6MWT was 4.74 longer than the control group (95% CI 0.81-8.67, P=0.02), with no significant heterogeneity (I^2 = 10%) (Figure 10). The improvement in the 6MWT distances in the burosumab group points to enhanced physical functioning and endurance in treated patients.

3.4.7 Height Z score

Changes in the Height Z score before and after treatment were assessed by seven studies (31, 32, 35, 38, 40–42) (Figure 11). The analysis showed no significant change in Height Z score (SMD 0.38, 95% CI −0.35-1.1, P=0.31) with high heterogeneity (I^2=96%), rendering the results inconclusive. The inconclusive results regarding changes in Height Z scores, accompanied by significant heterogeneity, suggest the need for further research to fully understand burosumab's impact on growth in XLH patients.

3.4.8 Subgroup analysis

Comparing burosumab to traditional phosphate and active vitamin D supplements in treating pediatric XLH patients is crucial due to burosumab's targeted mechanism of inhibiting FGF23, potentially offering more direct correction of the underlying phosphate wasting. This comparison is essential to assess burosumab's effectiveness in improving bone health, its convenience with less frequent dosing enhancing patient adherence, and its side effect profile relative to conventional treatments that often come with gastrointestinal issues and risk of secondary hyperparathyroidism. Moreover, understanding the long-term impacts on growth, skeletal abnormalities, and cost-effectiveness given burosumab's anticipated higher costs, is vital for providing evidence-based recommendations for managing XLH. To this end, we have performed a subgroup analysis of trials that contrast patients treated with burosumab against those receiving conventional therapy. The outcomes of this analysis could offer crucial insights for patients contemplating their future treatment choices (Figure 12).

3.4.8.1 1,25-$(OH)_2$D

The subgroup analysis of three studies (31, 32, 37) demonstrated a more significant increase in 1,25-$(OH)_2$D levels in the burosumab group compared to traditional therapies, with a SMD of 1.27 (95% CI 0.17-2.38, P=0.02). This significant improvement suggests burosumab's superior efficacy in correcting the aberrant vitamin D metabolism associated with XLH.

3.4.8.2 Serum Phosphorus

The analysis, incorporating data from six studies (31, 32, 35–38), showed a pronounced improvement in serum phosphorus levels in the burosumab-treated group, with an SMD of 2.43 (95% CI 1.49-3.37, P<0.00001). This finding highlights burosumab's potent effect on ameliorating hypophosphatemia, a hallmark of XLH.

3.4.8.3 TmP/GFR

Combining results from both randomized-control (32, 38) and cohort controls (31, 36, 37) revealed a substantial increase in TmP/GFR in the burosumab group, with an SMD of 3.14 (95% CI 2.29-3.99, P<0.00001). This indicates burosumab's effectiveness in

TABLE 1 Characteristics of included studies.

Imel, E. A. et al. (2019) (32)	
Methods	Study design: randomized control study Time frame: 2016 to 2019 Follow-up period: 140 weeks Excluded: 61
Participants	Country: International Setting: National multicentre study Children with XLH, serum phosphorus <3.0 mg/dL (<0.97 mmol/L) Number: 61 Mean age: 6.27 ± 3.307 (1 to 12) Sex(M/F): 27/34
Interventions	Active control(32) Multiple daily doses of oral phosphate and one or more daily doses of active vitamin D therapy, titrated and individualized by the investigator based on published recommendations during the Treatment Period (up to Week 64) During the Treatment Extension Period (Week 64 to Week 140), participants crossed over to receive a starting dose of SC burosumab 0.8 mg/kg Q2W Burosumab(29) 0.8 mg/kg starting dose, administered Q2W by SC injection during the Treatment Period (up to Week 64) During the Treatment Extension Period (Week 64 to Week 140), participants continued to receive a starting dose of SC burosumab 0.8 mg/kg Q2W
Outcomes	RGI-C RSS Total Score RGI-C Long Leg Score Height-For-Age Z-Scores Growth Velocity Z Score Serum Phosphorus $1,25(OH)_2D$ TmP/GFR Serum ALP PROMIS Pediatric Pain Interference, Physical Function Mobility and Fatigue Domain Scores FPS-R 6MWT Total Distance
Notes	Participants in Japan and Korea did not enter the Treatment Extension Period Primary outcome was RGI-C Global Score at Week 40
Whyte, M. P. et al. (2018) (42)	
Methods	Study design: cohort study Time frame: 2016 to 2019 Follow-up period: 160 weeks Excluded: 13
Participants	Country: USA Setting: University teaching hospital Children with XLH, Serum fibroblast growth factor 23 (FGF23) level > 30 pg/mL, Serum phosphorus < 3.0 mg/dL (0.97 mmol/L), Serum creatinine within age-adjusted normal range Number: 13 Mean age: 2.94 ± 1.146 (1 to 4) Sex(M/F): 9/4
Interventions	Burosumab subcutaneous (SC) injections every 2 weeks (Q2W) for a total of 160 weeks
Outcomes	Serum Phosphorus Number of Participants With Adverse Events (AEs), Treatment Emergent AEs (TEAEs), Serious TEAEs, and TEAEs Leading to Discontinuation RGI-C Score RSS Total Score RGI-C Lower Limb Deformity Score Recumbent Length/Standing Height Serum Alkaline Phosphatase
Notes	Primary outcomes were Change From Baseline at Week 40 in Serum Phosphorus and Number of Participants With Adverse Events (AEs), Treatment Emergent AEs (TEAEs), Serious TEAEs, and TEAEs Leading to Discontinuation One participant was withdrawn by subject
Linglart, A. et al. (2019) (41)	
Methods	Study design: cohort study Time frame: 2014 to 2018

(Continued)

TABLE 1 Continued

Linglart, A. et al. (2019) (41)	
	Follow-up period: 160 weeks Excluded: 52
Participants	Country: USA Setting: University teaching hospital Children with XLH, Serum phosphorus ≤ 2.8 mg/dL (0.904 mmol/L), Serum creatinine within age-adjusted normal range Number: 52 Mean age: 8.5 ± 1.87 (5 to 12) Sex(M/F): 24/28
Interventions	Burosumab Q2W(26) Burosumab SC injections every 2 weeks (Q2W). Dose is determined by the participant's weight and prescribed dose by their study doctor. Burosumab Q4W Then Q2W Burosumab subcutaneous (SC) injections every 4 weeks (Q4W). Dose is determined by the participant's weight and prescribed dose by their study doctor. Participants in Q4W were to switch to Q2W beginning with Week 64 dosing.
Outcomes	RSS Total Score Serum Phosphorus Serum $1,25(OH)_2D$ TmP/GFR RSS Knee Scores RSS Wrist Scores RGI-C Global Scores RGI-C Knee Scores RGI-C Wrist Scores Growth Velocity Growth (Standing Height, Sitting Height, Arm Length, Leg Length) 6MWT Distance POSNA-PODCI (Normative Score) Upper Extremity Scale Scores POSNA-PODCI (Normative Score) Transfer and Basic Mobility Scale Scores POSNA-PODCI (Normative Score) Sports/Physical Functioning Scale Scores POSNA-PODCI (Normative Score) Pain/Comfort Scale Scores POSNA-PODCI (Normative Score) Happiness Scale Scores POSNA-PODCI (Normative Score) Global Functioning Scale Scores FEP P1NP CTx ALP BALP Serum Pre-Dose Concentrations of burosumab Number of Participants With Treatment Emergent Adverse Events (TEAEs), Serious Adverse Events (SAEs) and Discontinuations Due to Adverse Events (AEs)
Notes	Primary outcome measures were: [Time Frame: Baseline, Week 40, 64, 160] Change From Baseline in RSS Total Score Over Time Change From Baseline in Serum Phosphorus Over Time Change From Baseline in Serum $1,25(OH)_2D$ Over Time Change From Baseline in TmP/GFR Over Time
Namba, N. et al. (2022) (37)	
Methods	Study design: cohort study Time frame: 2017 to 2022 Follow-up period: 121.7 weeks Excluded: 15
Participants	Country: Japan Setting: Hospital Children aged ≥ 1 and ≤12 years, Patients who have open growth plate, Willing to perform a self-administration of KRN23 and available to perform a self-administration, Diagnosis of XLH Number: 15 Mean age: 6.70 ± 3.20 (1 to 12) Sex(M/F): 2/13
Interventions	Burosumab start with 0.8 mg/kg, and adjusted based on serum phosphorus levels and any safety concerns (maximum 2 mg/kg)
Outcomes	[Time Frame: up to week 128] Number of subjects for each adverse events Percentage of subjects for each adverse events

(Continued)

TABLE 1 Continued

Namba, N. et al. (2022) (37)	
	Effect to body temperature Effect to pulse rate Effect to respiratory rate Effect to blood pressure Effect to 12-Lead Electrocardiogram Effect to Renal Ultrasound Effect to Echocardiogram Serum phosphorus concentration at each test time point $1,25(OH)_2D$ at each test time point Alkaline phosphatase at each test time point Urine phosphorus at each test time point Tubular reabsorption of phosphate at each test time point TmP/GFR at each test time point Change from baseline in serum phosphorus Change from baseline in $1,25(OH)_2D$ Change from baseline in alkaline phosphatase Change from baseline in urine phosphorus Change from baseline in tubular reabsorption of phosphate Change from baseline in TmP/GFR Improvement in Radiographic Global Impression of Change (RGI-C) global score Change from baseline on Rickets Severity Score (RSS) total score Change from baseline in the Six Minute Walk Test Change in height-for-age z-scores from baseline Serum KRN23 concentration Anti-KRN23 antibody
Notes	Primary outcome measures were: Number of subjects for each adverse events [Time Frame: up to week 128] Percentage of subjects for each adverse events [Time Frame: up to week 128]
Martín Ramos, S. et al. (2020) (36)	
ethods	Study design: cohort study Time frame: 2019 to 2020 Follow-up period: one year Excluded: 5
Participants	Country: Spain Setting: Hospital Patients younger than 18 years of age with XLH genetically confirmed and on treatment with burosumab for more than a year Number: 5 Mean age: 11.00 ± 3.847 (6 to 16) Sex(M/F): 2/3
Interventions	Burosumab one-year treatment with burosumab, injected subcutaneously at 0.8 mg/kg every 2 weeks
Outcomes	Serum phosphate Serum AP Serum $1,25(OH)_2D$ Serum PTH TRP TmP/GFR Height in cm Skeletal findings Dental abnormalities
Brener, R. et al. (2022) (35)	
Methods	Study design: cohort study Time frame: 2022 Follow-up period: three years Excluded: 10
Participants	Country: USA Setting: Pediatric Metabolic Bone Disease Unit in a tertiary medical center. Children with XLH Number: 10 Mean age: 8.80 ± 3.80 (4.3 to 15) Sex(M/F): 4/6

(Continued)

TABLE 1 Continued

Brener, R. et al. (2022) (35)	
Interventions	Burosumab SC injections Q2W The dose was adjusted (between 0.8 - 2mg/kg) to achieve a serum phosphorus level at the low end of the normal range for age and for healing the rickets
Outcomes	Serum phosphate Serum calcium Serum alkaline phosphatase Serum PTH Height, Weight, BMI Rickets severity score Pulp-coronal height ratio Pulp-coronal width ratio
Notes	Each visit included anthropometric measurements, physical examination, laboratory evaluation and imaging (left hand, wrists, knees and OPT). The routine laboratory evaluation at each time point included serum concentrations of phosphate, calcium, alkaline phosphatase and intact parathyroid hormone.
Kubota, T. et al. (2023) (39)	
Methods	Study design: cohort study Time frame: 2017 to 2020 Follow-up period: 114.8weeks (range 73.9 – 119.9) Excluded: 20
Participants	Country: Japan and South Korea Setting: hospital Children with XLH Number: 20 Mean age: 7.40 ± 3.40 (1 to 13) Sex(M/F): 6/14
Interventions	Burosumab SC injections Q2W A median dose of 17.36 mg (range 7.52 - 51.00 mg) every 2 weeks
Outcomes	Serum phosphate TmP/GFR Serum $1,25(OH)_2D$ Serum iFGF23
Notes	In the clinical development program of burosumab, self-administration was permitted and monitored in patients with XLH in two open-label, single-arm clinical studies conducted in Japan and South Korea
Paloian, N. J. et al. (2022) (38)	
Methods	Study design: control study Time frame: 2022 Follow-up period: 3.9 years (1.4 to 16.3) and 24 months Excluded: 12
Participants	Country: USA Setting: hospital Children with XLH Number: 12 Mean age: Age at XLH diagnosis: 1 (1 to 3) Age at initiation of burosumab: 6 (2 to 18) Sex(M/F): 4/8
Interventions	Conventional therapy Elemental phosphorus given four times daily and calcitriol given once or twice daily Elemental phosphorus: 20 - 30 mg/kg/day and calcitriol: 20 ng/kg/day Burosumab SC injections Q2W 0.8 mg/kg/dose rounded to the nearest 10 mg in patients <18 years of age and 1 mg/kg/dose rounded to the nearest 10 mg for patients 18 years old and greater
Outcomes	Serum phosphorus Serum alkaline phosphatase

(Continued)

TABLE 1 Continued

Paloian, N. J. et al. (2022) (38)	
	Serum intact PTH Urine FEphosphorus Urine TmP/GFR Urine Ca/Cr
Levy-Shraga, Y. et al. (2023) (40)	
Methods	Study design: control study Time frame: 2018 to 2021 Follow-up period: 3 years Excluded: 35
Participants	Country: Israel Setting: Hospital Children with XLH Number: 35 Mean age: 7.5 ± 4.4 (at burosumab initiation) (0.6 to 15.9) Sex(M/F): 18/17
Interventions	Burosumab SC injections Q2W Initially at a dose of 0.4 to 0.8 mg/kg of body weight, rounded to the nearest 10 mg.
Outcomes	iFGF23 Phosphorus Calcium Creatinine Alkaline phosphatase iPTH 25-(OH)D 1,25-$(OH)_2$ D Calcium/creatinine (urine) TRP TmP/GFR Rickets severity score
Notes	The dosage was increased stepwise according to laboratory results, consistent with clinical practice guidelines, up to a maximum dose of 2 mg/kg body weight or 90 mg. The study included all the patients who began treatment with burosumab between January 1, 2018, and January 1, 2021.
Ewert, A. et al. (2023) (31)	
Methods	Study design: control study Time frame: 2022 - 2023 Follow-up period: 12 months Excluded: 93 (Age < 12 y (n = 65); Age ≥ 12 y (n = 28))
Participants	Country: Germany Setting: Hospital Children and adolescents with XLH Number:93 Mean age:9.6 (5.0 - 12.3) Age < 12y 6.9 (3.4 - 9.7) Age ≥ 12y 13.7 (12.3 - 15.2) Sex(M/F): 34/59
Interventions	Burosumab SC injections Q2W Initial burosumab dose of 0.4 mg/kg body weight given every 2 weeks
Outcomes	Phosphate TmP/GFR TRP ALP PTH 25-(OH)D 1,25-$(OH)_2$ D $U_{Ca/Crea}$

(Continued)

TABLE 1 Continued

Ewert, A. et al. (2023) (31)	
Notes	Reduced fasting, age-related serum Pi levels, after a washout period of at least 7 days in patients on conventional treatment. Titration of burosumab dose in increments of 0.4 mg/kg body weight to raise fasting serum Pi levels within the lower end of the normal age reference range, with a maximum dosage of 2.0 mg/kg body weight (maximum dose 90 mg). Discontinuation of burosumab if fasting serum Pi level were above the upper normal limit (ULN). Prior to the study period, 33 (94%) patients received conventional therapy, namely oral phosphate supplement and alfacalcidol.

enhancing renal phosphate reabsorption, further supporting its therapeutic advantage in XLH management.

3.4.8.4 ALP Levels

The subgroup analysis of five studies (31, 32, 35, 37, 38) indicated a significant reduction in ALP levels for patients treated with burosumab, with an SMD of -2.43 (95% CI -3.93 to -1.19, P=0.0002). This outcome reflects burosumab's capacity to normalize bone turnover markers, suggesting improved bone metabolism in treated patients.

3.4.8.5 Height Z Score

The analysis of changes in Height Z score yielded inconclusive results. The SMD was 1.43 (95% CI -0.36 to 3.21, P=0.12), with high heterogeneity. This outcome signifies the complexity of assessing burosumab's impact on growth and necessitates further longitudinal studies to elucidate this aspect.

Given that RSS and 6MWT each have only a single valid study comparing burosumab to conventional therapy, subgroup analysis was not conducted for these two metrics.

The subgroup analysis elucidates burosumab's efficacy over conventional therapies across several key parameters, underscoring its potential to offer a more targeted and effective treatment for XLH. However, the variability in outcomes, especially regarding growth (Height Z score), underscores the necessity for ongoing research to fully understand burosumab's long-term benefits and implications in pediatric XLH treatment.

4 Discussion

Children with X-linked hypophosphatemia (XLH) often face significant challenges due to the debilitating effects of rickets and osteomalacia. These conditions not only compromise their physical well-being but also impact their quality of lives. While conventional treatments have included vitamin D metabolites and phosphate supplements, the emergence of burosumab has introduced a promising alternative in ameliorating the negative effects of XLH.

This meta-analysis comprehensively evaluated the efficacy of burosumab in the treatment of pediatric XLH patients. Ten high-quality clinical studies were systematically analyzed, revealing significant improvements in key parameters such as 1,25-$(OH)_2$D levels, serum phosphorus, TmP/GFR, RSS, ALP levels, and 6MWT performance (31, 32, 35–42). Despite limitations in some individual studies, the findings provide compelling evidence supporting burosumab as an effective treatment for XLH, offering significant benefits in various aspects of patient health and well-being.

The subgroup comparison between burosumab treatment and conventional therapy within the context of pediatric XLH patients reveals crucial insights into the effectiveness of these two approaches (31, 32, 35–38). As elucidated in this meta-analysis, the comparison consistently indicates that burosumab is superior to conventional treatments in increasing serum phosphorus levels and active vitamin D levels (31, 32, 37), enhancing renal phosphate reabsorption (31, 32, 36–38), and decreasing ALP levels (31, 32, 35, 37, 38), underscoring its potential to offer XLH patients a more effective and comprehensive treatment solution. However, limited data on RSS and 6MWT performance mean that further studies are needed to conclusively compare these treatments. This finding is crucial for clinicians and patients in deciding the most effective therapeutic approach for managing pediatric XLH.

One notable aspect illuminated by this analysis is the multifaceted impact of burosumab. Beyond improvement in biochemical markers, the drug demonstrates tangible benefits in the form of enhanced mobility, reduced bone deformities, and overall quality of life. This emphasizes the importance of evaluating XLH treatments beyond traditional biochemical markers, focusing also on functional and quality-of-life outcomes. Burosumab's influence on RSS (35, 37, 38, 40–42) and 6MWT (32, 37, 41) signifies this effective approach, addressing not only biochemical imbalances but also the functional limitations experienced by patients. This perspective aligns with the patient-centered care paradigm, emphasizing treatments that address both physiological and real-world challenges faced by pediatric XLH patients.

The XLH patients before burosumab treatment is generally poor due to the numerous physical and psychological challenges associated with the disorder, including chronic pain, skeletal deformities, and impaired mobility. Many patients require corrective orthopedic surgeries due to severe bone deformities, which add to the physical and emotional burden. Despite the promising results of burosumab therapy in treating XLH in biochemical markers, there is currently limited QOL data available on its use. Two reports indicate that burosumab has been effective in reducing bone pain and correcting skeletal deformities, significantly improving mobility and physical comfort, as well as enhancing happiness and life satisfaction for patients (28, 29). However, comprehensive data on how burosumab impacts patients' overall well-being, daily functioning, and social interactions are still needed. This lack of extensive QOL data highlights the need for further research to fully understand the therapy's benefits and drawbacks from the patients' perspectives. Understanding the effects of burosumab on QOL is crucial for optimizing treatment plans and ensuring that the therapy not only

TABLE 2 Quality assessment of the included studies based on the Newcastle-Ottawa scale (cohort studies).

	Selection[a]				Comparability[b]	Exposure[c]			
Author	Representativeness of the exposed cohort[e]	Selection of the non-exposed cohort[f]	Ascertainment of exposure[g]	Demonstration that outcome of interest was not present at start of study[h]	Comparability of cases and controls on the basis of the design or analysis[i]	Assessment of outcome[j]	Was follow-up long enough for outcomes to occur[k]	Adequacy of follow up of cohorts[l]	Score[d]
Linglart, A. et al. (2019) (41)	⋆	⋆	⋆	_	⋆	⋆	⋆	⋆	7
Namba, N. et al. (2022) (37)	⋆	⋆	⋆	_	⋆⋆	⋆	⋆	⋆	8
Whyte, M. P. et al. (2018) (42)	⋆	⋆	⋆	_	⋆	⋆	⋆	⋆	7
Martín Ramos, S. et al. (2020) (36)	⋆	⋆	⋆⋆	_	⋆	⋆	⋆	⋆	8
Brener, R. et al. (2022) (35)	⋆	⋆	⋆⋆	_	⋆	⋆	⋆	⋆	8
Kubota, T. et al. (2023) (39)	⋆	⋆	⋆	_	⋆	⋆	⋆	⋆	7
Ewert, A. et al. (2023) (31)	⋆	⋆	⋆	_	⋆⋆	⋆	⋆	⋆	8
Levy-Shraga, Y. et al. (2023) (40)	⋆	⋆	⋆	_	⋆	⋆	⋆	⋆	7

⋆:one point

aA maximum of one point for each item. [b]A maximum of two points for each item. [c]A maximum of one point for each item. [d]A maximum of nine points. [e](a)truly representative of the average XLH in the community⋆, (b) somewhat representative of the average XLHin the community⋆,(c) selected group of users eg nurses, volunteers,(d)no description of the derivation of the cohort. [f](a) drawn from the same community as the exposed cohort⋆, (b)drawn from a different source,(c)no description of the derivation of the non-exposed cohort. [g](a) secure record (eg surgical records) ⋆,(b) structured interview ⋆, (c) written self-report,(d)no description. [h](a) yes⋆, (b) no. [i](a) the exposures of interest (Burosumab and active control) were adjusted for one confounder (sex or age) ⋆, (b) the exposures of interest (burosumab and active controll) were adjusted for two or more confounders (sex or age and treatment compliance or severity of illness) ⋆⋆, c) no description. [j](a) independent blind assessment⋆, (b)record linkage⋆, (c) self-report, (d)no description. [k](a) yes (select an adequate follow up period for outcome of interest)⋆, (b) no. [l](a) complete follow up - all subjects accounted for ⋆, (b) subjects lost to follow up unlikely to introduce bias - small number lost - > 80% follow up, or description provided of those lost)⋆, (c) follow up rate < 80% and no description of those lost, (d)no statement.

TABLE 3 Quality assessment of the included studies based on the Newcastle-Ottawa scale (randomized control studies).

	Selection[a]				Comparability[b]	Exposure[c]			
Author	Is the case definition adequate?[e]	Representativeness of the cases[f]	Selection of Controls[g]	Definition of Controls[h]	Comparability of cases and controls on the basis of the design or analysis[i]	Ascertainment of exposure[j]	Same method of ascertainment for cases and controls[k]	Non-Response rate[l]	Score[d]
Imel, E. A. et al. (2019) (32)	⋆	⋆	⋆	_	⋆⋆	⋆	⋆	⋆	8
Paloian, N. J. et al. (2022) (38)	⋆	⋆	⋆	_	⋆⋆	⋆	⋆	⋆	8

⋆:one point

aA maximum of one point for each item. [b]A maximum of two points for each item. [c]A maximum of one point for each item. [d]A maximum of nine points. [e](a) yes, with independent validation ⋆, (b) yes, for example, record linkage or based on self-reports, (c) no description. [f](a) consecutive or obviously representative series of cases ⋆, (b) potential for selection biases or not stated. [g](a) community controls ⋆,(b) hospital controls, (c) no description. [h](a) no history of disease (endpoint) ⋆, (b) no description of source. [i](a) the exposures of interest (burosumab and active control) were adjusted for one confounder (sex or age) ⋆, (b) the exposures of interest (burosumab and active controll) were adjusted for two or more confounders (sex or age and treatment compliance or severity of illness) ⋆⋆, c) no description. [j](a) secure record (e.g., surgical records) ⋆, (b)structured interview where blind to case/control status ⋆, (c) interview not blinded to case/control status, (d) written self-report or medical record only, (e) no description. [k](a) yes ⋆, (b) no. [l](a) same rate for both groups ⋆, (b) non-respondents described, (c) rate different and no designation.

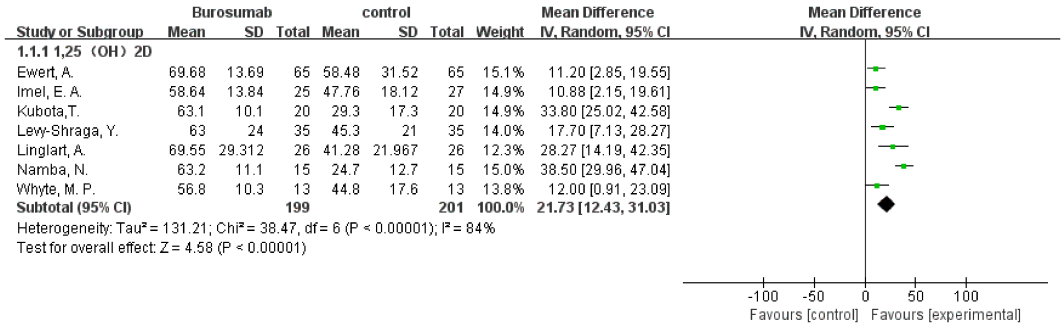

FIGURE 2
Meta-meta-analysis of the effects of burosumab on serum 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

addresses the clinical manifestations of XLH but also enhances the overall life satisfaction and daily functioning of those affected.

In addition to QOL concerns, several unknown clinical questions regarding its short-term effects need to be addressed through ongoing research and clinical practice. Immediate side effects, such as injection site reactions and hypersensitivity, require more comprehensive data across different age groups and patient populations. Additionally, the optimal dosage and administration schedule for various patient demographics need further refinement to ensure effective and safe treatment. Monitoring short-term biochemical responses, including changes in serum phosphate and ALP levels, is crucial for predicting treatment outcomes and adjusting therapy. While phosphate levels have been consistently used to monitor burosumab therapy and have shown significant improvement in this meta-analysis, recent findings suggest that ALP is a more stable and reliable marker. Many patients experience sustained growth and ALP normalization on burosumab treatment without achieving normal plasma phosphate concentration (31, 43). ALP provides a consistent biochemical indicator of bone activity, which allows for a more accurate and comprehensive assessment of the therapy's effectiveness in children. Evaluating the immediate impact on bone health markers, such as bone density, growth rates in children, and reductions in bone pain and fractures, is also essential. Assessing the short-term impact on patients' quality of life, including pain reduction, mobility, and daily functioning, provides a holistic view of its benefits. Understanding patient responses during the acute phase of burosumab treatment is important for setting patient expectations and managing care effectively. Addressing these questions through rigorous clinical trials will be crucial to fully understanding the short-term effects of burosumab therapy and optimizing its use in managing XLH.

Additionally, the meta-analysis raises critical questions about the long-term safety and efficacy of burosumab. While the analyzed studies provide encouraging short-to-medium-term outcomes, the long-term impacts remain uncertain. Longitudinal studies tracking patients over several years are necessary to ascertain the sustainability of burosumab's benefits. Furthermore, the potential for adverse effects in the context of prolonged monoclonal antibody therapy warrants careful consideration, demanding a comprehensive risk-benefit analysis.

Moreover, the findings of this meta-analysis accentuate the importance of individualized treatment approaches (41). XLH is a heterogeneous disorder, exhibiting significant variability in its clinical manifestations. Personalized approaches tailoring burosumab therapy to the specific needs of each patient is crucial. Personalized approaches taking into account individual factors such as age, disease severity, and comorbid conditions, could enhance therapeutic outcomes and minimize risks. There was one study that assessed the variance in burosumab treatment efficacy among children and adolescents. The findings indicated that there was no discernible difference in burosumab's impact on both age groups when indices like ALP levels, RSS value, serum phosphate, and

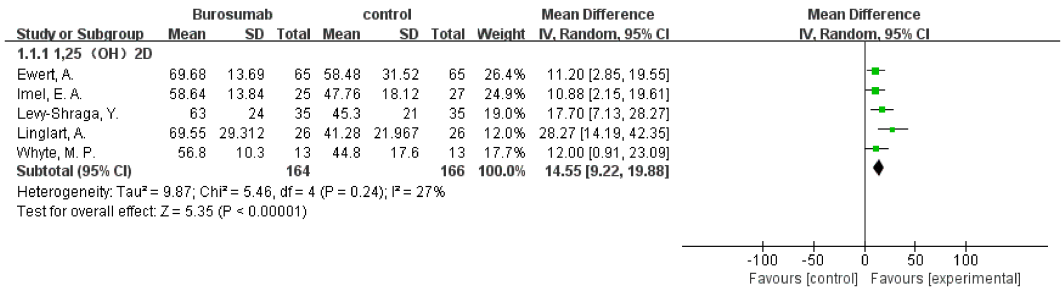

FIGURE 3
Sensitivity analysis of the effects of burosumab on serum 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D). The bottom row describes a combined overall effect of treatment, which is similar to previous result.

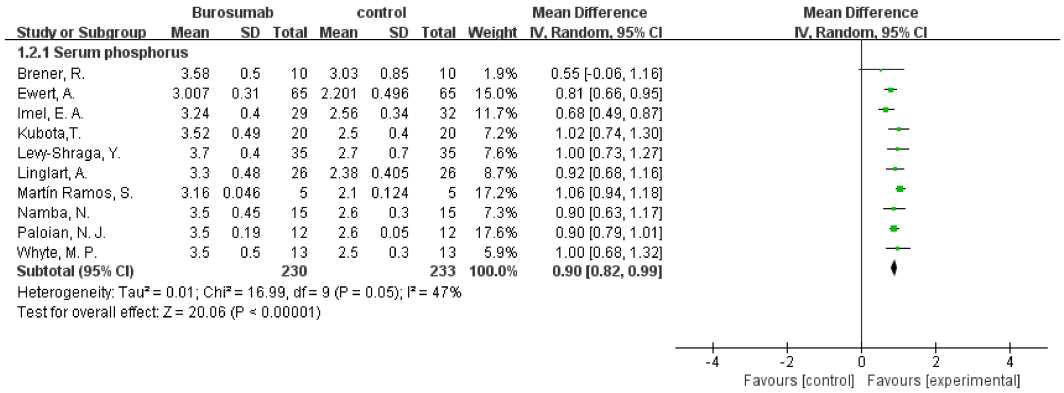

FIGURE 4
Meta-meta-analysis of the effects of burosumab on serum phosphorus. The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

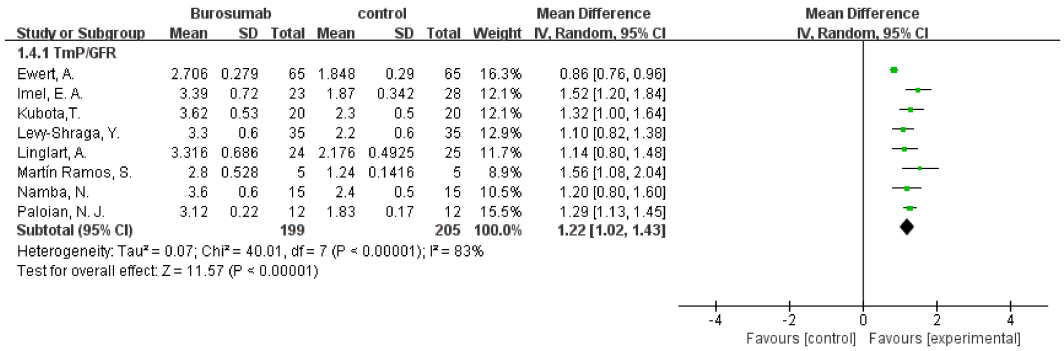

FIGURE 5
Meta-meta-analysis of the effects of burosumab on renal tubular maximum reabsorption rate of phosphate to glomerular filtration rate (TmP/GFR). The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

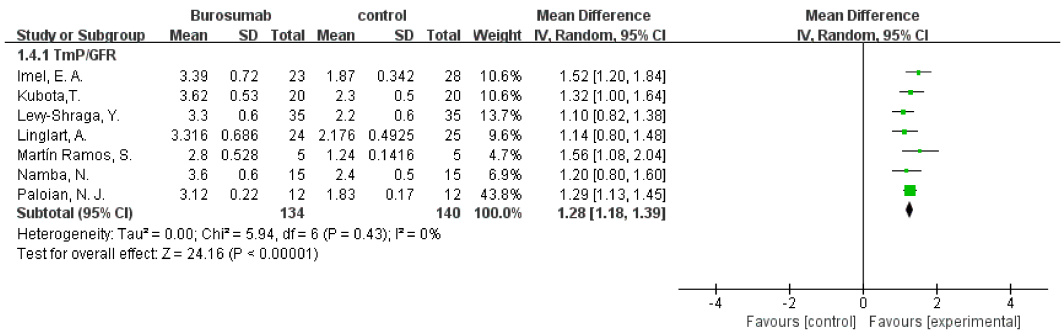

FIGURE 6
Sensitivity analysis of the effects of burosumab on renal tubular maximum reabsorption rate of phosphate to glomerular filtration rate (TmP/GFR). The bottom row describes a combined overall effect of treatment, which is similar to previous result.

TmP/GFR returned to normal (31). However, compared to younger children, adolescents require a lower unit dosage of burosumab.

There is evidence of sexual dimorphism in the severity of XLH, with males often exhibiting more severe symptoms than females. This difference is particularly noticeable in skeletal and dental impairments, aligning with the notion that males tend to have more severe mineralization defects (5, 30). The severity in males is thought to be influenced by variations in sex hormones, physical activity, and other factors (44). Burosumab has shown effectiveness in improving biochemical markers and physical symptoms in both males and females with XLH (37, 40). Similar improvements in serum phosphate levels, active vitamin D levels, renal phosphate

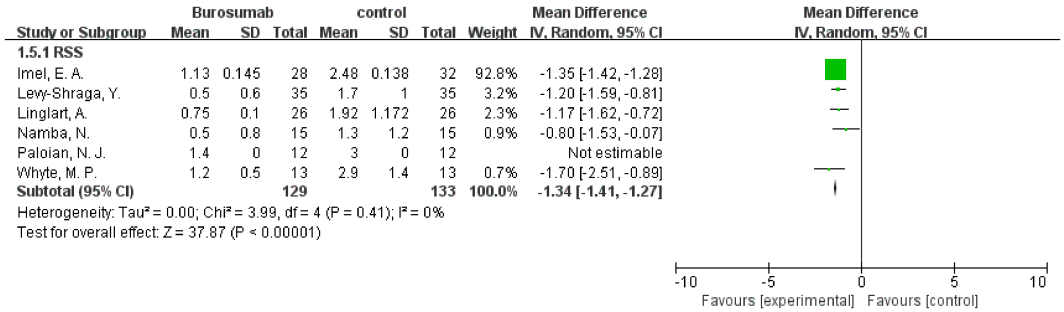

FIGURE 7
Meta-meta-analysis of the effects of burosumab on rickets severity score (RSS). The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

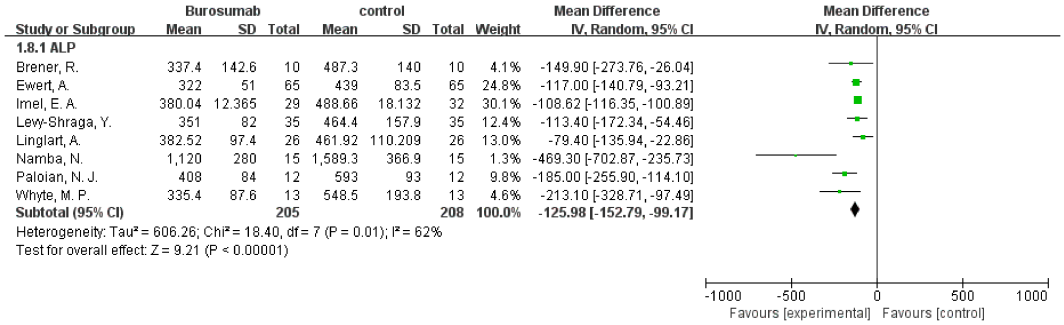

FIGURE 8
Meta-meta-analysis of the effects of burosumab on serum alkaline phosphatase (ALP). The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

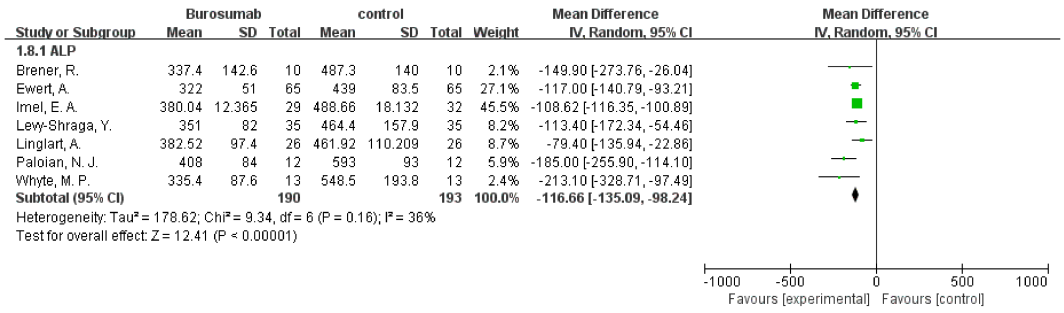

FIGURE 9
Sensitivity analysis of the effects of burosumab on serum alkaline phosphatase (ALP). The bottom row describes a combined overall effect of treatment, which is similar to previous result.

reabsorption, as well as physical symptoms were observed in both males and females. More detailed studies focusing on long-term outcomes and direct comparisons between males and females are needed to fully understand the nuances of burosumab's effectiveness across different patient groups. Such research is crucial to optimize treatment strategies and ensure that all patients, regardless of sex, achieve the best possible outcomes.

According to clinical practice guidelines for pediatric X-linked hypophosphatemia in the era of burosumab, it is widely endorsed an initial dosage of 0.8 mg/kg of body weight (which is changed to 0.4 mg/kg in Europe), rounded to the nearest 10 mg (maximum dose 90 mg), administered subcutaneously every 2 weeks (45). Once burosumab therapy commences, fasting serum phosphate levels should be monitored every 4 weeks for the initial 3 months. Dose

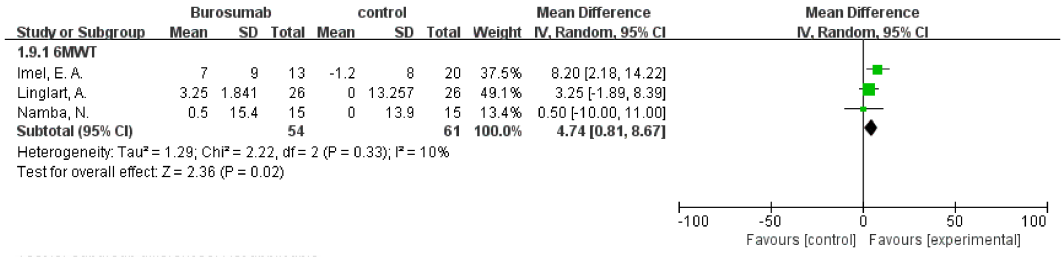

FIGURE 10
Meta-meta-analysis of the effects of burosumab on 6-minute walking test (6MWT). The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

adjustments are made based on changes in serum phosphate levels and other parameters, aiming to maintain them within the normal range for the child's age. For instance, if fasting serum phosphate falls below the normal range on two consecutive occasions (4 weeks apart), the dose should be increased. Conversely, if the level exceeds the normal range, the dose should be withheld and later resumed at a reduced level once it falls below the normal range. In relevant articles analyzed, Brener et al. tailored the dose (ranging from 0.8-2 mg/kg) to attain a serum phosphorus level at the lower limit of the normal range for age and facilitate rickets healing (35). Ewert et al. observed significant variations in the final weight-based burosumab doses among children aged 1 to 12, with an interquartile range of 0.72 to 1.41 mg/kg, yet failed to establish a notable correlation between these doses and other parameters (31). Notably, our findings indicate that dosage adjustments are common in most studies, and even the exhaustion of the maximum recommended dosage may not always lead to normalization of serum phosphorus level and TmP/GFR, implying that dosage variations stem from diverse patient conditions.

There is evidence that burosumab is also effective in adult XLH patients. In a double-blind, placebo-controlled phase 3 trial, adults with XLH received subcutaneous injections of 1:1 burosumab 1 mg/kg (n = 68) or placebo (n = 66) every 4 weeks. The trial demonstrated that burosumab increased renal phosphate reabsorption and normalized serum phosphate levels throughout the dosing interval in symptomatic adults with XLH by binding to excess circulating FGF23 (46). 1,25$(OH)_2$D and TmP/GFR concentrations were higher in the burosumab group compared to the placebo group. Improvements in phosphate metabolism were accompanied by significant reductions in stiffness, increased body function, and decreased pain, which may be expected to improve mineralization and restore normal bone physiology. Additionally, another trial of burosumab treatment in adults demonstrated that burosumab can bring about a possible positive remodeling balance in which the serum markers of bone turnover were improved (47). The results in the 6MWT were used to evaluate exploratory efficacy endpoints for mobility and they were returned to normal levels after burosumab treatment. It also suggested that the efficacy of burosumab can lasts for more than 3 years on average, with no evidence of diminished or impaired clinical response after reintroduction of the drug after treatment interruption.

The cost-benefit balance between burosumab and conventional therapy is an important consideration in real-life practice. Burosumab therapy is more than 100 times higher in cost than conventional therapy, with an annual expense of approximately $160,000 per patient for children and $200,000 per patient for adults (49). The decision to use burosumab over conventional therapy involves evaluating these significant cost differences against the potential for better long-term outcomes and reduced complications with burosumab.

In conclusion, burosumab represents a significant advancement in treating pediatric XLH, yet the treatment landscape for this rare disorder is still evolving. Future research should focus on unraveling

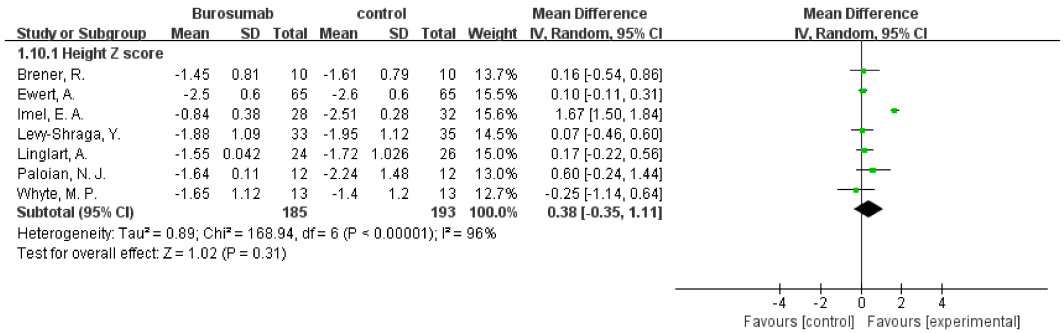

FIGURE 11
Meta-meta-analysis of the effects of burosumab on Hight Z score. The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

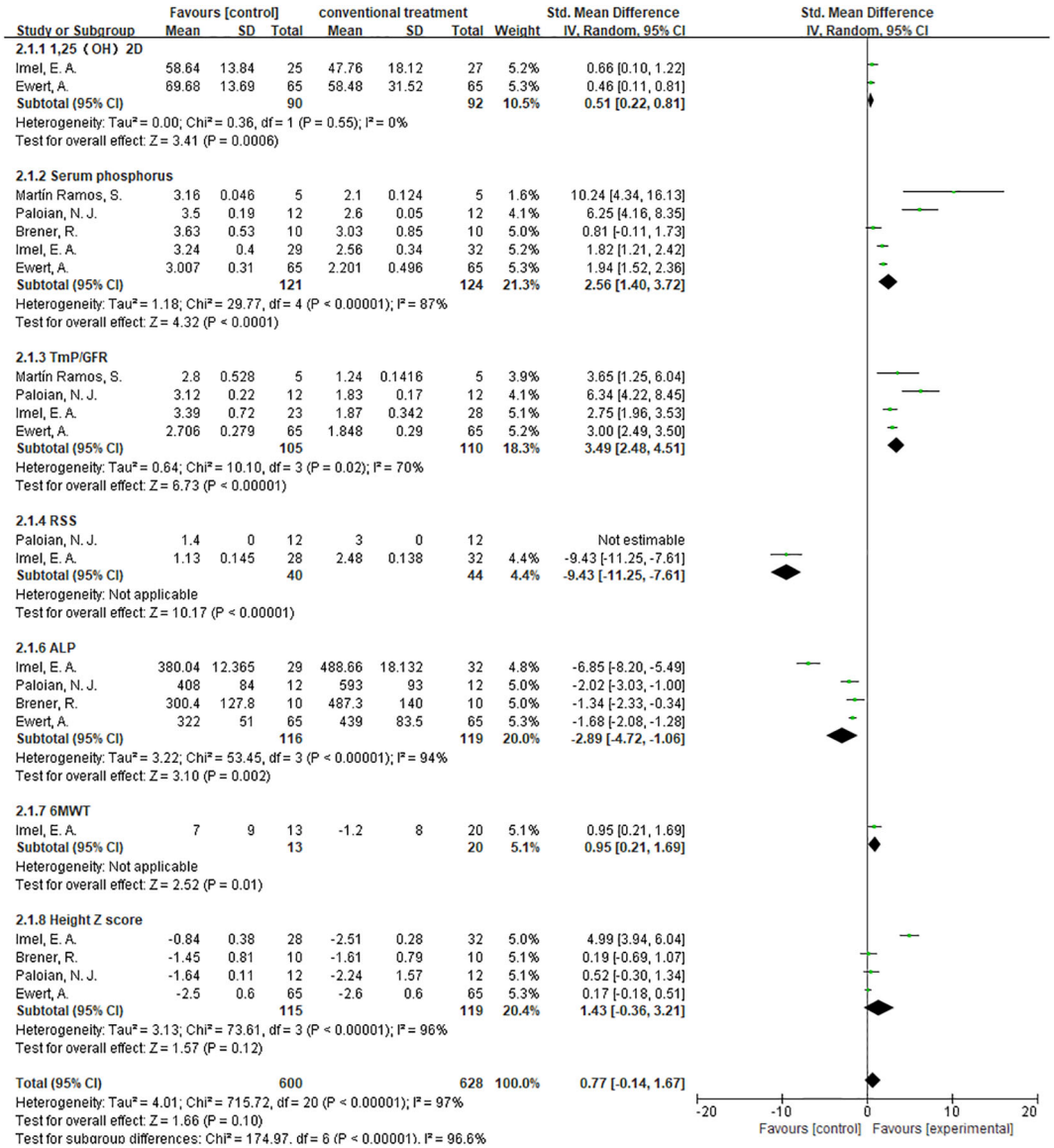

FIGURE 12
Meta-meta-analysis of the effect between burosumab and conventional therapy. The indexes include 1,25-dihydroxyvitamin D (1,25-$(OH)_2$D), serum phosphorus, renal tubular maximum reabsorption rate of phosphate to glomerular filtration rate (TmP/GFR), rickets severity score (RSS), serum alkaline phosphatase (ALP), 6-minute walking test (6MWT) and Hight Z score. The bottom row describes a combined overall effect of treatment which random-effects models were used to estimate.

the molecular mechanisms of XLH and the specific action mode of burosumab. Additionally, exploring combinational therapies integrating burosumab with other targeted treatments could represent the next frontier in XLH management. A multidisciplinary approach, integrating genetics, molecular biology, and clinical expertise, is essential to further improve outcomes for individuals with XLH, aiming for minimal life impact from the disorder.

Author contributions

KW: Writing – original draft, Data curation, Formal analysis, Investigation, Validation. RZ: Data curation, Formal analysis, Investigation, Validation, Writing – original draft. ZC: Data curation, Formal analysis, Investigation, Validation, Writing – original draft. YB: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition. QH: Writing – original draft, Conceptualization, Funding acquisition, Supervision, 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.

Publisher's note

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Minimizing unnecessary brain magnetic resonance imaging in pediatric endocrinology: a retrospective cohort analysis

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Background: Brain magnetic resonance imaging (MRI) is mandatory or highly recommended in many pediatric endocrinological conditions to detect causative anatomic anomalies and rule out neoplastic lesions. However, MRI can also show findings associated with the underlying clinical condition, as well as unrelated "incidentalomas". These latter findings are often abnormalities with a high incidence in the general population for which there is no clear literature regarding their management, especially in pediatric patients. The present study aimed to evaluate the number of unnecessary performed MRIs in pediatric endocrinology.

Methods: Retrospective analysis on 584 MRI scans performed in 414 patients (254 growth hormone deficiency, 41 other causes of short stature, 116 central precocious puberty).

Results: The MRI scans were completely normal in 67% of the individuals, and the prevalence of individuals who underwent more than one MRI was 18%, with no significant differences among the groups. The overall prevalence of incidentalomas was 17%. Among 170 repeated MRI scans, 147 (86%) were not required according to a dedicated protocol. Only five patients (four GHD, one Noonan) correctly repeated the MRI. All the repeated MRI scans did not reveal any progression in the findings. If we include the MRIs performed in cases of OCSS other than Noonan syndrome (n=32) and girls with CPP older than 6 years (n=89), an additional 121 MRIs could have been avoided, leading to a total number of unnecessary MRIs to 268 (46%).

Conclusions: Only a few specific neuroimaging findings in endocrinologic pediatric patients warrant further investigation, while too often repeated imaging is carried out unnecessarily. We advocate the importance of guidelines to reduce costs for both the healthcare system and patients' families, as well as to alleviate physical and psychological distress for patients and caregivers.

KEYWORDS

brain magnetic resonance imaging, incidentaloma, growth hormone deficiency, central precocious puberty, follow-up

Introduction

Brain magnetic resonance imaging (MRI) is mandatory or highly recommended to detect causative anatomic anomalies and rule out neoplastic lesions in many pediatric endocrinological conditions, such as central precocious puberty (CPP) (1) and growth hormone deficiency (GHD) (2) or other causes of short stature (OCSS) in which recombinant human growth hormone (rhGH) is prescribed such as Noonan syndrome (3). Often, previously unsuspected anomalies and malformations are found at brain MRI and sometimes these findings are completely unrelated to the clinical aspect for which the radiological examination was requested (4, 5). In the latter case, such a finding is referred to as "incidentaloma". Due to the lack of clear pediatric guidelines for the management and follow-up of these radiological findings, sometimes the use of radiological exams might become excessive and even disproportionate to the patient's medical needs. In a previous study, we reported the suggested management of the most frequent brain findings found in pediatric patients affected by GHD and CPP (6), which would result in a reduction of not only economic but also physical and psychological implications. The present study aimed to evaluate the number of unnecessary performed MRIs in pediatric endocrinology. Our goal was to highlight that, based on the current literature, most of the follow-up brain MRIs are probably not required and that only a few neuroimaging findings are worth subsequent investigations.

Materials and methods

This is a retrospective cohort study on children who had a brain MRI with pituitary protocol performed at the Institute for Maternal and Child Health IRCSS "Burlo Garofolo" in Trieste, Italy, from 01/07/2007 to 31/12/2020, because of a diagnosis of CPP, GHD, or OCSS conditions in which rhGH is prescribed (i.e., Turner syndrome, chronic renal insufficiency, Prader–Willi syndrome, SHOX deficiency, children born small for gestational age [SGA] without catch-up growth, Noonan syndrome, and idiopathic short stature [ISS] cleared by the regional GH commission) (7). GHD was diagnosed in children with growth defects (height ≤−3 SDS; or height ≤−2 SDS and growth velocity ≤−1 SDS; or growth velocity ≤−2 SDS or ≤−1.5 SDS after 2 consecutive years, even without short stature) based on positive arginine and insulin stimulation tests; a GH peak below 8 μg/L in both tests was considered as pathological (8); sex steroid priming was not used since it is not recommended in our national guidelines; and patients with already known multiple pituitary deficits were excluded from the study. PPC was diagnosed in patients with secondary sexual characteristics before 8 years of age in girls and 9 years in boys with a GnRH test showing an LH peak >5 mU/mL and/or an LH/FSH peak ratio >1. In accordance with our internal protocol, all OCSS patients received an MRI scan prior to commencing rhGH therapy to rule out any preexisting brain tumors.

Patients with previously known abnormal brain MRI scans requiring follow-up (n=8) were excluded from the study. The "G2 clinico" platform (management system specialist activities) was employed to access all patients' data. Information retrieved included date of birth, sex, indication for brain MRI (GHD, OCSS, CPP), date of brain MRI, and found abnormalities (if any). If multiple MRIs were performed, the number of MRIs, any changes in the findings, and the date of the last MRI were recorded.

Brain MRI with gadolinium-based contrast agents and with specific sequences for the hypothalamic–pituitary region was performed with a 1.5 Tesla Ingenia MR scanner (Philips Healthcare, Best, The Netherlands). Imaging in static mode with axial T2-, T1- and FLAIR (fluid-attenuated inversion recovery)-weighted sequences of the whole brain and subsequent thin-layer evaluation targeted to the pituitary gland in the sagittal and coronal planes, with T2- and T1-weighted sequences, were performed before and after administration of a full-dose contrast agent. Coronal scans allowed visualization of the pituitary gland, pedicle, chiasm, and parasellar regions whereas sagittal images were more suitable for evaluation of the midline plane. The acquisition procedure lasted an average of 20 min–30 min, and sedation was considered as a protocol in children <8 years of age (between 7 and 8 years was evaluated on a case-by-case basis). All the MRI scans were reviewed by an expert pediatric radiologist (FMM) with more than 15 years of experience.

The guidelines for the management and follow-up of MRI brain findings were first introduced in January 2021 (6). The following findings were considered as "alterations with definite or possible clinical/anatomical significance" (ADPCAS): adenohypophysis hypoplasia, pituitary stalk interruption syndrome (PSIS), ectopic neurohypophysis, complete or partial empty sella; Rathke cleft cyst (RCC), pituitary adenoma, craniopharyngiomas (or other tumors in the hypothalamus–pituitary region), and Arnold-Chiari type I. The following findings were included within the group "alterations without clinical significance/incidentalomas": arachnoid cyst, pineal cyst, choroid plexus cysts, vascular abnormalities, and increased pituitary volume. Other findings with low frequency (n ≤ 4) and not related to GHD and CPP were grouped as "other". The following findings were considered as "alterations worthy of radiological follow-up": (1) craniopharyngioma or other tumors (follow-up after surgery ± radio/chemotherapy according to the oncological guidelines); (2) pituitary adenoma: if symptomatic, surgical or medical therapy + MRI follow-up according to the oncological guidelines; if asymptomatic and ≥10 mm, MRI once per year for 3 years and then every 1–2 years, if asymptomatic and ≥5 mm (<10 mm), a single MRI after 1 year; (3) RCC: if symptomatic, radiologic follow-up for at least 5 years postsurgery; if asymptomatic and >5 mm, MRI at 1, 3, and 5 years (regardless of characteristics); (4) arachnoid cyst, if large and in high-risk regions; (5) pineal cysts, if >14 mm and/or with an abnormal radiological pattern or clinical symptoms.

Ethical Committee approval was not requested since General Authorization to Process Personal Data for Scientific Research Purposes (Authorization no. 9/2014) declared that retrospective archive studies that use ID codes, preventing the data from being traced back directly to the data subject, do not need ethics approval (9). Informed consent was signed by parents at the first visit, in which they agreed that "clinical data may be used for clinical research purposes, epidemiology, the study of pathologies and training, to improve knowledge, care and prevention". All data were collected in an anonymous database.

All statistical analyses were conducted with JMP™ (version 16.1.0, SAS Institute Inc., Cary, NC, United States). Descriptive statistics was used to describe data. Continuous variables were expressed as median with interquartile range, minimum, and maximum. Categorical data were expressed as percentages (%). For the comparison of continuous variables between more than two groups, the Kruskal–Wallis test was used. The chi-square test was used to compare categorical variables between the groups. Statistical significance was considered for p-values <0.05.

The study has been reported in line with the STROBE statement for observational studies (10).

Result

During the study period, a total of 584 MRI scans were performed on 414 patients: 257 GHD, 41 OCSS, 116 CPP. In the OCSS group, 16 were SGA (of which 3 Silver–Russell syndrome), 9 were Noonan syndrome, 7 were ISS, 3 were Turner syndrome, 3 were SHOX deficiency, 2 were IRC, and 1 was Prader–Willi syndrome.

Clinical and imaging features of the entire cohort and the three groups are presented in Table 1. Age at presentation was lower in CPP and OCSS (median 8.7 and 9.1 years, respectively) compared with GHD (median 11.5 years) (p<0.01), whereas CPP had a higher prevalence of women (83%) compared with GHD (37%) and OCSS (46%) (p<0.01). In the CPP group, 7 out of 96 women (7%) were diagnosed below the age of 6 years.

MRI scan was completely normal in 67% of the individuals (n=276) (Figure 1), with no significant differences among groups (GHD 65%, OCSS 68%, CPP 70%, p=0.63), whereas in 33% of the cases, a finding was reported (Table 1). ADPCAS were found in 16% (n=66) of the entire cohort (Figure 1): the GHD group had a higher prevalence (21%), compared with OCSS (7%) and CPP (9%) (p<0.01); in particular, PSIS (n=13), empty sella (n=8), adenohypophysis hypoplasia (n=8), and ectopic neurohypophysis (n=3) were only found in the GHD group (Table 1). Arnold–Chiari type 1 malformation, pituitary adenoma, and adenohypophysis hypoplasia were also detected in CPP, whereas RCC were found in both CPP and OCSS (1 Noonan, 1 ISS) patients, with no significant differences among groups (Table 1). No craniopharyngiomas were detected during the study period, but a dysgerminoma was found in a patient with GHD and a dysembryoplastic neuroepithelial tumor (DNET) in a patient with Noonan syndrome.

There was no significant difference in the median age between GHD patients with ADPCAS (11.3 years [6.7; 13.7]) and those without ADPCAS (11.6 years [8.57; 13.16]) (p=0.82). Similarly, the median age of CPP patients with APCAS (8.2 years [7.9; 9.2]) was not significantly different from those without APCAS (8.7 years [8.0; 9.3]) (p=0.39).

The overall prevalence of incidentalomas was 17% (Figure 1)—22% if we consider also those who had both incidentalomas and ADPCAS (n=92)—with no significant differences among groups (p=0.79). OCSS had a higher prevalence (21%) of "other" findings compared with CPP (7%) and GHD (6%): most of them were related to the underlying syndrome (Table 1).

The prevalence of individuals who performed more than one MRI was 18% (n=73), with no significant differences among diagnostic groups (GHD 19%, OCSS 10%, CPP 18%, p=0.37) (Table 2); the prevalence of patients with ADPCAS that performed more than one MRI (57%) was significantly higher than those without ADPCAS (10%, p<0.01). The median number of MRIs was 3, with a maximum of 11 in GHD, 7 in OCSS, and 5 in CPP, and the median distance from the first to the last MRI was 1.6 years (Table 2). The number of MRIs in patients with ADPCAS (median 4 [IQR 2;5], max 11) was significantly higher than in patients without ADPCAS (median 2 [IQR 2;3], max 6) (p<0.01).

According to our guidelines (6), only in five patients (7% of those with more than one performed MRI), four with GHD, and one with Noonan syndrome, a follow-up was required: two Rathke cleft cysts >5 mm, one non-functioning pituitary adenoma >10 mm, one dysembryoplastic neuroepithelial tumor, and one dysgerminoma. No patients with CPP or OCSS would have required a follow-up MRI. Details are reported in Table 3.

Overall, out of 170 repeated MRI scans, 147 (86%) were unnecessary, as none of these scans showed any progression of

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TABLE 1 Clinical and imaging features of the entire cohort and the three groups (* and in bold: alterations worthy of radiological follow-up.

	Total (n=414)	GHD (n=257)	OCSS (n=41)	CPP (n=116)	p
Age at presentation (years) (median, IQR)	9.7 (7.9;12.5)	11.5 (8.2;13.2)	9.1 (5.9;11.7)	8.7 (8.0;9.3)	**<0.01**
Female (n, %)	210 (51%)	95 (37%)	19 (46%)	96 (83%)	**<0.01**
Completely normal MRI	276 (67%)	167 (65%)	28 (68%)	81 (70%)	0.63
Any finding at MRI	138 (33%)	90 (35%)	13 (32%)	35 (30%)	
Alterations with definite or possible clinical/anatomical significance (ADPCAS)					
Overall	66 (16%)	52 (20%)	3 (7%)	11 (9%)	**<0.01**
• Arnold-Chiari type 1	17 (4%)	12 (5%)	–	5 (4%)	0.46
- <7 mm	14 (3%)	9	–	5	
- ≥7 mm	3 (<1%)	3	–	–	
• Rathke cleft cyst (RCC)	15 (4%)	9 (4%)	2 (5%)	4 (3%)	0.90
- Asymptomatic <5 mm	13	7	2	4	
- Asymptomatic ≥5 mm*	**2**	**2**	–	–	
- Symptomatic*	–	–	–	–	
• Pituitary stalk interruption syndrome (PSIS)	13 (3%)	14 (5%)	–	–	**0.02**
• Pituitary adenoma	10 (2%)	6 (2%)	–	4 (3%)	0.65
- <5 mm	9	5	–	4	
- 5 mm–10 mm*	–	–	–	–	
- >10 mm*	**1**	**1**	–	–	
• Empty sella	8 (2%)	9 (4%)	–	–	0.08
- Partial	8	8	–	–	
- Complete	–	–	–	–	
• Adenohypophysis hypoplasia	8 (2%)	8 (3%)	–	2 (2%)	0.08
• Ectopic neurohypophysis	3 (<1%)	3 (2%)	–	–	0.39
• Tumors*	**2 (<1%)**	**1 (<1%)**	**1 (<1%)**	–	0.15
• Septo-optic dysplasia	1 (<1%)	1 (<1%)	–	–	0.73
Incidentalomas					
Overall	92 (22%)	55 (21%)	10 (24%)	27 (23%)	0.86
• Arachnoid cyst	22 (5%)	15 (6%)	2 (5%)	5 (4%)	0.82
- Small cyst	22	15	2	5	
- Large cyst or localization at risk of hydrocephalus*	–	–	–	–	
• Pineal cyst	18 (4%)	15 (6%)	–	3 (3%)	0.13
- <14 mm, without abnormal radiological pattern, without clinical symptoms	18	15	–	3	
- >14 mm and/or with an abnormal radiological pattern or clinical symptoms*	–	–	–	–	
• Vascular anomalies	11 (3%)	4 (2%)	2 (5%)	5 (4%)	0.20
- Developmental venous anomalies	11	5	3	5	
- Cavernomas and arteriovenous malformations	–	–	–	–	
• Increased hypophyseal volume	11 (3%)	6 (2%)	–	5 (4%)	0.29

(Continued)

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TABLE 1 Continued

	Total (n=414)	GHD (n=257)	OCSS (n=41)	CPP (n=116)	p
Incidentalomas					
• Choroid plexus cyst	8 (2%)	4 (2%)	–	4 (3%)	0.30
• Other	25 (6%)	10 (4%)	7 (17%)	8 (7%)	**<0.01**

CPP, central precocious puberty; GHD, growth hormone deficiency; MRI, magnetic resonance imaging; OCSS, other causes of short stature)

findings. Considering an estimated cost of 450 euros for each MRI (of which 46.15 euros are paid by the patient's families), 66,150 euros could have been saved for unnecessary tests during the study period (6,785.05 by the families and 59,365.95 by the National Health System).

If we include the MRIs performed in cases of OCSS other than Noonan syndrome (n=32) and girls with CPP older than 6 years (n=89), an additional 121 MRIs could have been avoided. This brings the total number of unnecessary MRIs to 268 (46%) and the potential savings to 130,950 euros 12,368.20 euros by families and 108,231.80 euros by the National Health System). These figures do not include the costs associated with procedural sedations in younger children.

Discussion

Brain MRI is essential in many pediatric endocrinological conditions, such as GHD or CPP. However, it is not uncommon (13%–18%) (11, 12) to identify "incidentalomas", incidental findings serendipitously diagnosed in a patient undergoing imaging for an unrelated reason (13). Even the majority of alterations with definite or possible clinical or anatomical relevance (ADPCAS) do not progress over time and thus would not need radiological follow-up (6). In our previous paper, we emphasized that there was a lack of guidelines regarding the management of these findings in the pediatric population, often leading to repeated MRI scans, excessive and disproportionate to the patient's needs (6), potentially leading to significant resource expenditure and patient anxiety (14). To mitigate this risk, we have summarized the optimal management strategies for the most frequently identified alterations in patients with GHD and CPP. These strategies could not only reduce costs but also alleviate physical and psychological implications (6). Moreover, performing a brain MRI without a proper indication also raises ethical issues; written informed consent should be obtained after a detailed explanation of the reasons and the risks and benefits (15).

In this retrospective study, we evaluated the number of repeated MRIs performed and estimated the number of avoidable MRIs before the introduction of guidelines for the management and follow-up of brain MRI findings in January 2021 (6). During the study period, we identified a total of 584 MRI scans that were performed on 414 patients with GHD, CPP, or OCSS. The MRI scan was completely normal in two-thirds of the patients, and we did not observe significant differences among the three diagnostic groups (GHD, CPP, OCSS). These results confirm those obtained in many other studies on the topic, where normal MRI represents the most common outcome in children diagnosed with GHD and CPP (16–19).

Nevertheless, one-third of young individuals undergoing a brain MRI will have findings in the report that need explanation and may require follow-up, potentially causing additional anxiety for the family (14). Half of these individuals (17% of the entire cohort) had an "incidentaloma," with no significant differences in prevalence among groups. Although the number of patients with incidentalomas who underwent follow-up MRIs was significantly lower than those with ADPCAS (10% vs. 57%), and patients with

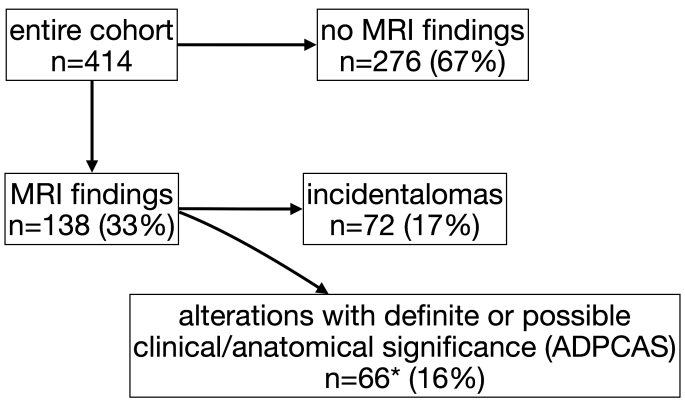

FIGURE 1
Flow diagram of study participants and magnetic resonance imaging (MRI) findings (*includes 20 patients whit also "incidentalomas").

TABLE 2 Data regarding repeated imaging (CPP, central precocious puberty; GHD, growth hormone deficiency; MRI, magnetic resonance imaging; OCSS, other causes of short stature) .

	Total (n=414)	GHD (n=257)	OCSS (n=41)	CPP (n=116)	p
Individuals with more than one MRI performed (n, %)	73 (18%)	48 (19%)	4 (10%)	21 (18%)	0.37
No. of MRI when >1 (median, IQR; max)	3 (2;4–max 11)	3 (2;4–max 11)	3 (2;6–max 7)	3 (2;3–max 5)	0.69
Distance from first to last MRI (years) (median, IQR)	2.0 (0.7;4.2)	2.7 (0.7;4.9)	1.9 (0.7;4.3)	1.0 (0.5;2.1)	0.11
Number of MRI with required follow-up (n, %)	5 (1%)	4 (2%	1 (2%)	–	0.33

incidentalomas underwent fewer follow-up MRIs compared with those with ADPCAS (median 2 vs. 4), they all performed multiple MRI without a clear indication.

The prevalence of ADPCAS was significantly higher in children with GHD (20%) compared with those with CPP and OCSS (9% and 7%, respectively). PSIS, empty sella, ectopic neurohypophysis, and septo-optic dysplasia were observed exclusively in cases of GHD (2, 20). On the other hand, Arnold-Chiari type 1 malformation (21), pituitary adenoma (22), and adenohypophysis hypoplasia (18) were also detected in CPP patients, whereas RCCs were also found in both CPP and OCSS patients (one Noonan, one ISS), as previously reported (23, 24). No craniopharyngiomas were detected during the study period, despite GHD or CPP often being its first sign of presentation, and MRI is strongly recommended by guidelines to rule out neoplastic lesions (18–20, 25). However, we did identify a dysgerminoma in a patient with GHD and a DNET in a patient with Noonan syndrome. This latter finding represents a rare intracranial tumor, already described in patients with Noonan syndrome (26). Although the median age at GHD diagnosis might seem higher than expected (27, 28), it is consistent with other Italian reports (29, 30), suggesting a possible delay in referrals and diagnosis in Italy. Furthermore, the median age of GHD patients with ADPCAS (indicating definite GHD) was similar to that of patients without ADPCAS (who might be classified as having short stature unresponsive to stimulation tests) (31), corroborating the fact that there is no an error in diagnosis.

Overall, 147 out of 170 repeated MRI scans (86%) were unnecessary, since none of these findings would have needed a follow-up according to current literature, and none showed any progression of findings. An appropriate evidence-based follow-up was carried out in only five cases (32–37). Therefore, adherence to the criteria outlined in our study for follow-up could have reduced costs (66,150 euros) and psychological stress for the patients that imaging studies inevitably entail. According to the guidelines we proposed in our previous study (6), brain MRI findings that did not require radiological follow-up could be adequately managed with clinical and laboratory monitoring alone. Among ADPCAS, the management of an empty sella warrants further discussion, as there is a significant gap in evidence-based guidelines. Some authors advocate for radiological follow-up due to the theoretical risk of progression (38, 39). However, given the very low incidence of neuroradiological progression, which correlates with hormonal deterioration, clinical and laboratory monitoring alone may be sufficient for these patients (39). If new signs, symptoms, or hormonal changes suggestive of progression occur, an MRI evaluation becomes necessary. Our data have revealed that all findings that underwent more than one MRI remained stable over time, suggesting, therefore, that extensive follow-up is not necessary when they do not initially present insidious characteristics.

In addition to reconsidering repeated MRIs, it is important to question the necessity of performing an MRI initially. While the indication for neuroimaging in GHD is clear—to identify anatomical anomalies that may explain the etiology and to exclude neoplastic lesions that could contraindicate rhGH therapy (2)—there are still controversies about the necessity of performing MRI scans on all children with CPP. The likelihood of finding significant intracranial abnormalities in girls over 6 years old is low, although not zero (1, 40). In our cohort, we did not find any neoplastic lesions in CPP patients. By excluding girls over the age of six from MRI screenings, we could have avoided 89 tests.

It is not uncommon to perform an MRI before starting treatment with rhGH in non-GHD patients to rule out neoplastic lesions (3, 41, 42), even though no published guidelines support the routine use of brain MRI in these cases. It should be noted that in conditions where rhGH is indicated, such as Turner syndrome, Noonan syndrome, or *SHOX* deficiency, GHD is typically not

TABLE 3 Clinical and imaging features of lesions with indicated radiological follow-up (DNET, dysembryoplastic neuroepithelial tumor; GHD, growth hormone deficiency; RCC, Rathke's cleft cyst) .

Patient	Sex	Diagnosis	Age at diagnosis	Finding	Number of MRIs performed
1	F	GHD	12.1 years	Dysgerminoma	9
2	M	Noonan syndrome	13.5 years	DNET	7
3	F	GHD	12.1 years	Pituitary adenoma >10 mm	4
4	F	GHD	12.1 years	RCC > 5 mm	4
5	F	GHD	11.8 years	RCC > 5 mm	3

excluded, although coincidences have been reported (43–45). Furthermore, the rate of abnormal MRI findings is similar in short children with normal GH responses and normal IGF-1 levels compared with children with GHD (46). Currently, evidence does not support the use of MRI of the pituitary region in short children born SGA without GHD (47), whereas MRI is recommended in children with Noonan syndrome (3), due to their higher risk of developing tumors, including brain tumors, compared with the general population (48, 49). In our cohort, a DNET was found in a patient with Noonan syndrome. Therefore, we recommend a precautionary brain MRI before initiating rhGH therapy in NS patients to exclude any preexisting brain tumors. In other patients with OCSS, an MRI might not be necessary, unless they present neurological symptoms or signs of hypopituitarism. With this approach, we could have spared an additional 32 MRIs.

Overall, we could have spared 268 MRIs (46% of the total number), resulting in potential savings of more than 130,000 euros, not including the costs for procedural sedations. By fine-tuning our internal protocol, we aim to further reduce the number of MRIs, including repeat scans, in the coming years.

While our study offers valuable insights, it is not without limitations. Primarily, it relies on retrospective data. However, we followed meticulous steps to mitigate potential biases. All available data were gathered to minimize selection bias, and to ensure objectivity, every MRI was scrutinized by an expert pediatric radiologist, mitigating reporting bias. Additionally, being a single-center study, there is a possibility that our findings may not fully represent broader populations. Nevertheless, the consistency of our results with prior literature suggests potential generalizability. Lastly, a notable constraint arises from the absence of a "healthy" control group among the pediatric population undergoing neuroimaging solely for pituitary disorders. This limitation is inherent in many similar studies in the literature exploring brain alterations in children with pituitary-related diseases (4). On the other hand, to our knowledge, this is the first study evaluating the follow-up of MRI findings in children with endocrine disorders. There is a lack of guidelines on incidentalomas and MRI brain abnormalities in the pediatric population. Therefore, our study can support many pediatricians who daily find themselves having to manage such clinical conditions.

With this study, we confirm that only a few neuroimaging findings in pediatric patients warrant further investigation, and we highlight that too often investigations are carried out unnecessarily. We advocate the importance of being familiar with guidelines for prescribing these exams and managing these findings to reduce costs for both the healthcare system and patients' families, as well as to alleviate physical and psychological distress for patients and caregivers.

Data availability statement

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

Ethics statement

Ethical Committee approval was not requested since General Authorization to Process Personal Data for Scientific Research Purposes (Authorization no. 9/2014) declared that retrospective archive studies that use ID codes, preventing the data from being traced back directly to the data subject, do not need ethics approval. All data were collected in an anonymous database. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements because informed consent was signed by parents at the first visit, in which they agreed that "clinical data may be used for clinical research purposes, epidemiology, the study of pathologies and training, to improve knowledge, care and prevention".

Author contributions

MM: Data curation, Investigation, Writing – original draft, Writing – review & editing. FM: Data curation, Investigation, Writing – original draft, Writing – review & editing. FB: Conceptualization, Validation, Writing – original draft, Writing – review & editing. GTa: Validation, Writing – original draft, Writing – review & editing. EF: Validation, Writing – original draft, Writing – review & editing. EB: Resources, Validation, Writing – original draft, Writing – review & editing. GTo: Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Supervision, 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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Case report: Novel germline c.587delA pathogenic variant in familial multiple endocrine neoplasia type 1

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Multiple Endocrine Neoplasia type 1 (*MEN1*) is a rare genetic disease, characterized by co-occurrence of several lesions of the endocrine system. In *MEN1*, the pathogenic *MEN1* gene mutations lead to the Abnormal expression of menin, a critical tumor suppressor protein. We here reported a case of a 14-year-old male with insulinoma and primary hyperparathyroidism. Genetic testing demonstrated a novel heterozygote variant c.587delA of MEN1, resulting in the substitution of the 196th amino acid, changing from glutamic acid to glycine, followed by a frameshift translation of 33 amino acids. An identical variant was identified in the proband's father, who was further diagnosed with hyperparathyroidism. To the best of our knowledge, this is the first report of MEN1 syndrome caused by the c.587delA MEN1 variant. Observations indicated that, despite sharing the same MEN1 gene change, family members exhibited diverse clinical phenotypes. This underscored the presence of genetic anticipation within the familial context.

KEYWORDS

multiple endocrine neoplasia type 1, MEN1 gene, menin, pathogenic variant, pediatric endocrinology

1 Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) is a rare genetic disease, characterized by co-occurrence of several lesions of the endocrine system (1, 2). Prevalence of MEN1 is 3–10/100,000 (1). MEN1 most frequently involves the parathyroids and pancreas, pituitary gland, adrenal glands, lungs, and thymus in descending order of occurrence. The extent of

penetrance also depends on the specific endocrine organ affected. The disease's penetrance increases with age, beginning as early as 10 years old and reaching near-universal levels by 60 years of age (3). Transmission is autosomal dominant, linked to heterozygous inactivating variants of the MEN1 gene, located at 11q13 (1, 4). Spanning 9 kilobases with 10 exons, the MEN1 gene encodes the protein menin, which consists of 610 amino acids and plays a crucial role in various cellular mechanisms through interactions with numerous partners (1, 5). Functioning as a tumor suppressor gene, MEN1 gene changes result in neuroendocrine tumors (NETs) via the "double hit" hypothesis proposed by Knudson (1).

According to the diagnostic criteria established in 2012 (2), MEN1 can be diagnosed by one of three criteria: on clinical criteria, by presence of at least 2 major MEN1 lesions; on familial criteria, by presence of a MEN1 lesion in a first-degree relative of an individual presenting clinical MEN1; on genetic criteria, in an individual presenting a pathogenic or probably pathogenic variant of the MEN1 gene, whether symptomatic or not. Patients presenting with clinical MEN1 or suspected MEN1 according to the criteria should undergo genetic analysis to screen for MEN1 gene abnormality. Any pathogenic or probably pathogenic variant of the MEN1 gene thus identified must be checked on a second sample (1). Family members of patients clinically diagnosed with MEN1 who develop a MEN1-associated tumor meet the criteria for a familial MEN1 diagnosis (2).

This case report describes a family with MEN1 due to a novel heterozygous pathogenic variant,c.587delA. This variant leads to the substitution of the 196th amino acid, changing from glutamic acid to glycine, followed by a frameshift that results in the translation of an additional 33 amino acids before premature termination. Moreover, this case highlights the variability in disease phenotypes among family members carrying the same gene change.

2 Case presentation

The index case, a 14-year-old male, presented with recurrent seizure-like episodes for over 2 years. Subsequent to an episode of unrousability upon awakening, fasting blood glucose was found to be 2.09 mmol/L (normal range 3.9-5.9), insulin levels at 23.96 μU/ml (normal range 1.5-15), and C-peptide at 0.972 nmol/L (normal range 0.48-0.78). Enhanced abdominal Magnetic Resonance Imaging(MRI) scanning indicated a nodular lesion in the anterior part of the pancreatic body, suggestive of a neuroendocrine tumor (Figure 1). Physical examination upon admission did not reveal any abnormalities.

3 Diagnostic assessment

On January 29, 2019, the patient underwent distal pancreatectomy and partial pancreatectomy under general anesthesia. Pancreatic pathology reported tumor cells positive for PCK (dot-like+), CgA (+), Syn (+), CD56 (+), ATRX (+), Rb (sporadically+), P53 (+, 5%), with a Ki-67 positivity rate of 5%-10%, diagnosing it as a Grade 2 neuroendocrine tumor (NET G2). Immunohistochemistry showed positivity for insulin; glucagon, gastrin, and somatostatin were negative. The final diagnosis was an insulinoma. Post-surgery, fasting blood glucose levels ranged from 4.28 to 5.02 mmol/L, and fasting insulin levels varied from 6.28 to 13.18 μU/ml, with no post-operative seizure-like episodes.

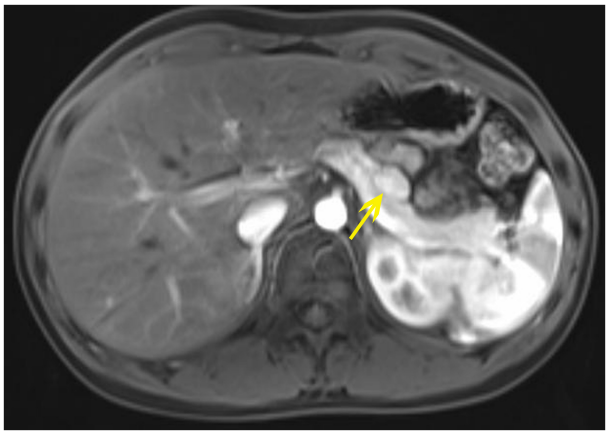

FIGURE 1
The abdominal MRI with contrast of the proband shows: a nodular lesion approximately 1.7x1.2 cm in size located in the anterior part of the pancreatic body, displaying slightly prolonged T1 and T2 signals. Diffusion is suspiciously restricted, with marked enhancement post-contrast, consistent with the enhancement of the pancreatic parenchyma.

Calcium, phosphate, and PTH levels were normal during the hospital stay. But during follow-up, elevated blood calcium, PTH levels, and decreased phosphate levels were observed. Blood calcium levels were monitored at 2.62-2.81 mmol/L (normal range 2.1-2.7 mmol/L), phosphate at 1.07-1.33 mmol/L (normal range 0.81-1.45 mmol/L), and PTH at 9.85-11.35 pmol/L (normal range 1.60-6.90 pmol/L), with a 24-hour urinary calcium excretion of 12.94 mmol/24h (normal range 2.5-7.5) and urinary phosphate of 27.0 mmol/24h (normal range 22-48). Bone metabolism markers indicated a significant increase in bone turnover. The patient experienced no symptoms of polydipsia, polyuria, nausea, vomiting, abdominal pain, back pain, hematuria, or bone pain. Parathyroid ultrasound revealed: bilateral deep parathyroid solid nodules. Parathyroid single photon emission computed tomography/computed tomography (SPECT/CT) hybrid Imaging: four parathyroid glands were visualized, and there was no significant increase in parathyroid technetium-99m methoxyisobutylisonitrile (99mTc-MIBI) scan uptake (Figure 2).

Bone density was within the normal range for the same age group. Urinary system ultrasound showed no urinary tract stones. On July 3, 2019, under general anesthesia, "neck exploration + total parathyroidectomy + partial autotransplantation of the parathyroid gland" was performed. Paraffin pathology diagnosis of the upper left, lower left, and upper right parathyroid tissues indicated hyperplasia. The surgery was successful, with preoperative PTH measured at 11.44 pmol/L and postoperative PTH at 30 min measured at 1.15 pmol/L. Postoperative treatment included calcium supplementation and vitamin D. Four weeks

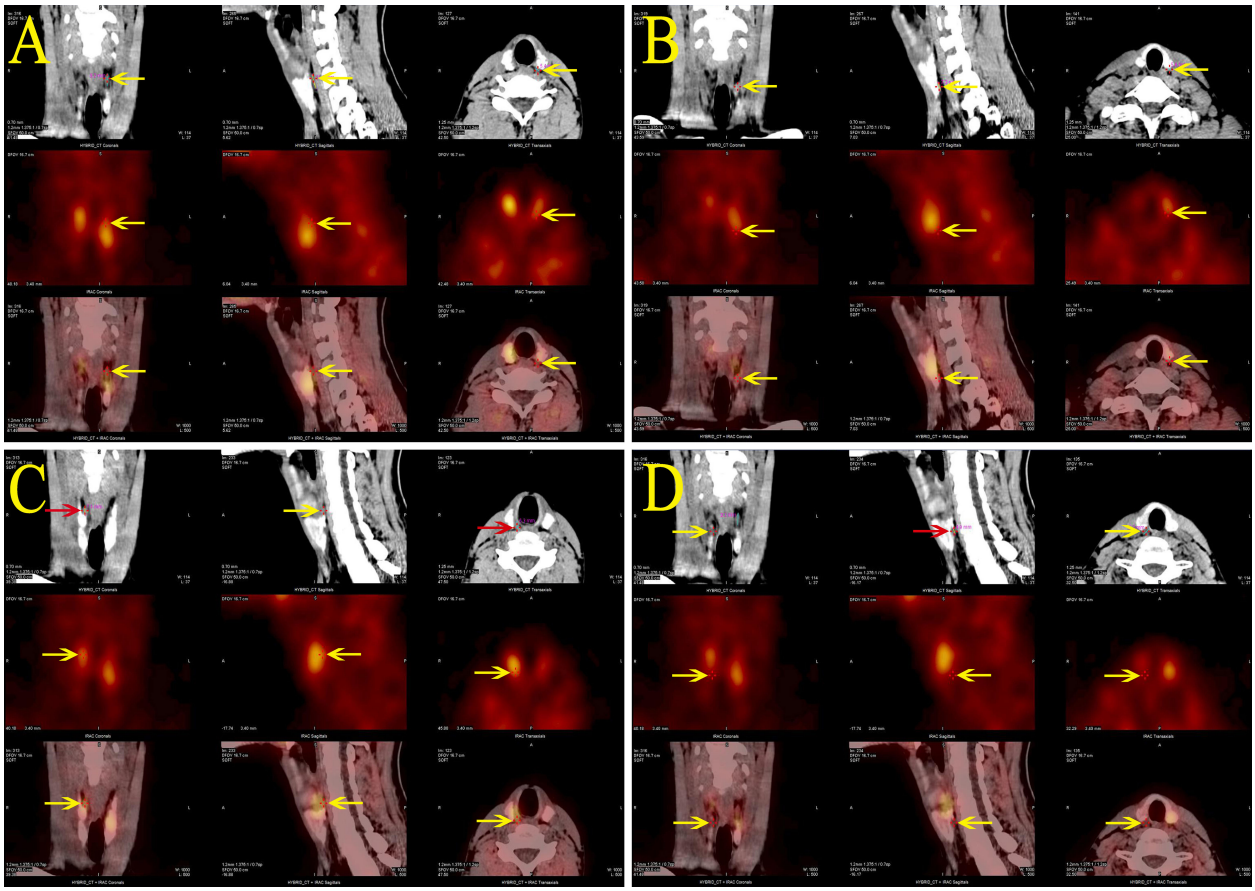

FIGURE 2
Parathyroid SPECT/CT hybrid imaging of the proband: four areas of low-density shadows located posteriorly in the upper portions of both thyroid lobes **(A, C)**, the posterior inferior part of the left lobe **(B)**, and the posterior middle part of the right lobe **(D)**, suggesting a parathyroid tissue origin. However, none of these areas demonstrated significant MIBI uptake.

postoperatively, PTH was 1.98 pmol/L, blood calcium 2.33 mmol/L, and phosphate 1.7 mmol/L. Medications were gradually discontinued, and follow-up to date shows normal blood calcium, phosphate, PTH levels, with no hypoglycemia or seizure episodes, and no symptoms or biochemical markers of other MEN1-associated systemic diseases.

Given the patient's concurrent presentation with an insulinoma and hyperparathyroidism, we conducted screening for MEN1-associated disease phenotypes and genetic testing. The patient's gastrin levels, pituitary and target gland axis hormones, blood and urine catecholamines and their metabolites, aldosterone-renin-angiotensin system, complete blood count, liver and kidney function tests, urinalysis, and tumor markers (AFP, CEA, CA19-9, CYFRA21-1, NSE) were all within normal ranges. Ultrasounds of the kidneys, adrenal glands, and scrotum, as well as chest and head CT scans, showed no abnormalities. Pituitary MRI revealed no mass lesions.

Deepened whole-exome sequencing of the patient identified a genetic variant c.587delA on chromosome 11 (chr11:64575445) (Figure 3A), resulting in an amino acid sequence change of p.Glu196GlyfsTer33(NM_130803). This denotes a base A deletion at position 587 in the coding region, leading to the substitution of the 196th amino acid from glutamic acid to glycine, followed by a frameshift translation of 33 amino acids before translation termination. The total sequencing depth was 144, with a variant depth of 84, indicating heterozygosity. According to the 2015 ACMG guidelines, the variant is classified as likely pathogenic: PVS1 (loss-of-function variant likely results in gene inactivation) + PM2 (MAF <0.005, considered a low-frequency variant).

MEN1 gene testing was performed on the index case's parents. The father carried the same gene change, also as a heterozygote (Figure 3B), and the mother was wild type (Figure 3C). The father, a 43-year-old male with a history of recurrent back pain for over 6 years, had undergone multiple procedures for bilateral ureteral stones, including extracorporeal shock wave lithotripsy and transurethral ureterolithotomy, with postoperative analysis confirming the stones as calcium oxalate. Physical examination revealed no positive findings. Further tests showed parathyroid hormone (PTH) at 24.28 pmol/L, blood calcium at 2.77 mmol/L, serum inorganic phosphorus at 0.67 mmol/L, and fasting blood glucose at 5.67 mmol/L. A 24-hour urine electrolyte analysis indicated calcium at 4.38 mmol/24h and phosphorus at 16.20 mmol/24h. Bone metabolism markers suggested a significant increase in bone turnover. Gastrin, pituitary and target gland axis hormones, blood and urine catecholamines and their metabolites, aldosterone-renin-angiotensin system, fasting blood glucose,

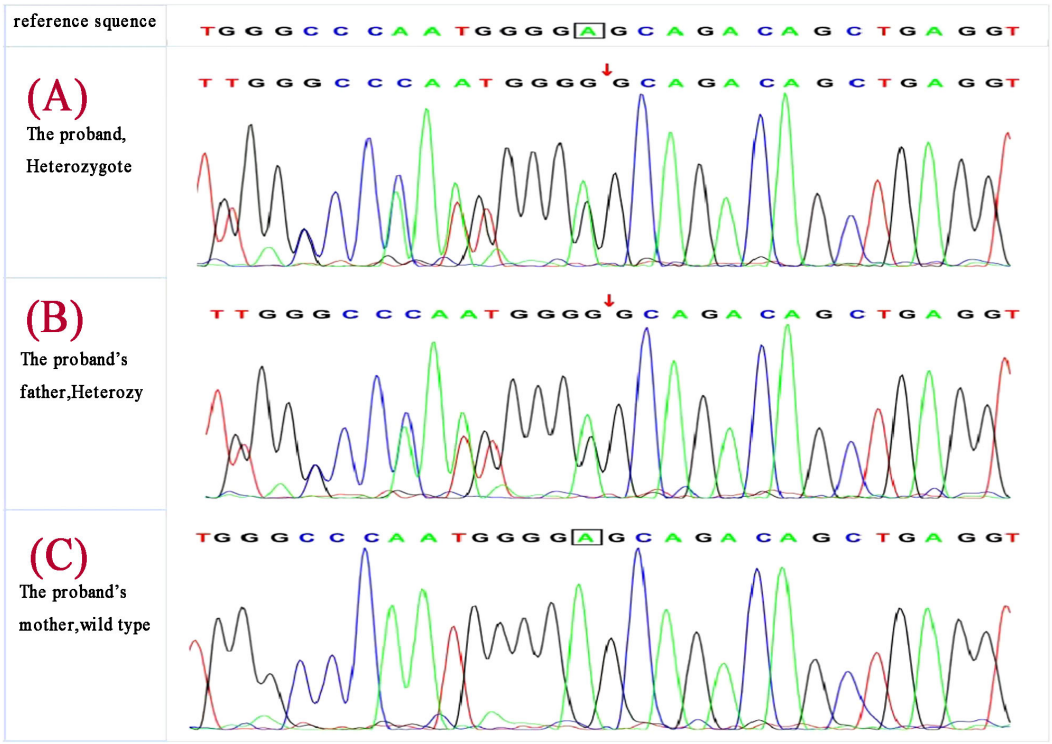

FIGURE 3
Deepened whole-exome sequencing identified a genetic variant c.587delA on chromosome 11 (chr11:64575445) in the proband **(A)**, the proband's father carried the same gene change **(B)** ,and the mother was wild type **(C)**.

insulin, C-peptide, complete blood count, liver and kidney function, urinalysis, and tumor markers were all normal. Enhanced MRI of the pancreas and pituitary MRI showed no definitive abnormalities. Parathyroid SPECT/CT hybrid imaging revealed mild increased MIBI uptake in a nodule behind the lower part of the right thyroid lobe, suggesting parathyroid tissue origin (Figure 4). On July 9, 2019, a neck exploration and resection of the right lower parathyroid adenoma were performed, in line with the patient's preference against subtotal parathyroidectomy. Pathology indicated adenomatous hyperplasia of the right lower parathyroid, with immunohistochemistry showing PTH (+), CD56 (-), Syn (+, partial), CgA (+), and a Ki-67 positivity rate of approximately 1-2%, special staining for reticular fibers (Foot) was positive. Postoperative reevaluation showed blood calcium at 2.51 mmol/L and phosphate at 0.95 mmol/L. Follow-up to the present day, February 18, 2024, shows the father with normal blood calcium, phosphate, PTH levels, no further episodes of urinary tract stones, and no emergence of other MEN1-associated disease phenotypes.

The proband has no siblings, and his grandparents and father's siblings were unwilling to undergo genetic testing, opting only for screening of blood glucose, calcium, phosphate, and PTH, all of which were normal. In this case, the c.587delA gene variant in the index case as a heterozygote shows co-segregation with the phenotype and genotype of the father, consistent with autosomal dominant inheritance. The c.587delA variant is confirmed as the pathogenic gene variant causing familial MEN1. In summary, we have compiled a comprehensive table detailing the therapeutic interventions and laboratory test results pertaining to the proband and his father throughout the course of their diagnosis and treatment. This information can be found in Table 1 of the supplementary materials.

3.1 Material and methods

Whole-exome sequencing was performed with the IDT xGen Exome Research Panel v1.0 for exome capture, followed by sequencing on the Illumina NovaSeq 6000 series sequencer. The sequencing coverage for targeted sequences was no less than 99%.

4 Discussion

The MEN1 gene encodes the menin protein, which is a scaffold protein that is involved in histone modification and epigenetic gene regulation (6), playing a role in tumor suppression associated with MEN1. Menin interacts with over 50 different known proteins, influencing various cellular processes like cell cycle progression, DNA repair, and transcriptional regulation. It interacts with transcription factors and chromatin-modifying proteins, affecting pathways like TGF-β/BMP, nuclear receptors, Wnt/β-catenin, and Hedgehog, crucial for gene expression regulation. Loss of menin affects these interactions, impeding these signaling pathways and their anti-proliferative effects (7).The inactivation of menin due to

genetic variants can lead to the loss of its tumor-suppressive function. Pathogenic variants of MEN1 usually have a truncating effect on menin, with loss of the tumor-suppressing function and an increased risk of developing cancer (8, 9).

More than 1,300 variants in the MEN1 gene have been identified, distributed throughout the open reading frame, primarily in the coding exons as well as in intron sequences, without significant clustering or notable hot spots (9, 10). Approximately 69% of these germline variants in MEN1 are deemed pathogenic, leading to early truncation of menin. This is predominantly due to frame-shift variants (42%) and nonsense variants (14%), along with exon region deletions resulting from splicing defects (10.5%) and extensive deletions (2.5%) (8).

If a gene change that has not been previously reported is considered pathogenic, confirming its pathogenicity often requires demonstrating the same variant in another affected first-degree relative (11).The clinical diagnosis of MEN1 relies on detecting neoplastic disorders in at least two organs typically involved, such as the parathyroid glands, the anterior pituitary, and the pancreas. For our young male patient, pancreas was the initial sign of MEN1, followed by the discovery of Insulinoma and primary hyperparathyroidism. Genetic analysis identified a novel heterozygous c.587delA MEN1 variant, results in the substitution of the 196th amino acid from glutamic acid to glycine, followed by a frameshift translation of 33 amino acids before translation termination. Genetic validation demonstrated the same variant was also detected in his father, who was further diagnosed with hyperparathyroidism. In the case of the patient's father, identical MEN1 symptoms as observed in his son were identified, allowing for the validation of the variant's pathogenicity in a novel context. It was thought that such c.587delA MEN1 variant and frame-shift variants resulted in aberrant translation of menin, thereby impairing its tumor-suppressing function, and then leading to the occurrence of MEN1.

Observations indicated that, despite sharing the same MEN1 gene change, the family members exhibited diverse clinical phenotypes. The father developed the condition only in adulthood, exhibiting Solitary parathyroid adenoma, while the son experienced severe hypoglycemia symptoms due to an insulinoma before the age of 12, and subsequently developed hypercalcemia caused by hyperplasia of multiple parathyroid glands. This underscored the presence of genetic anticipation

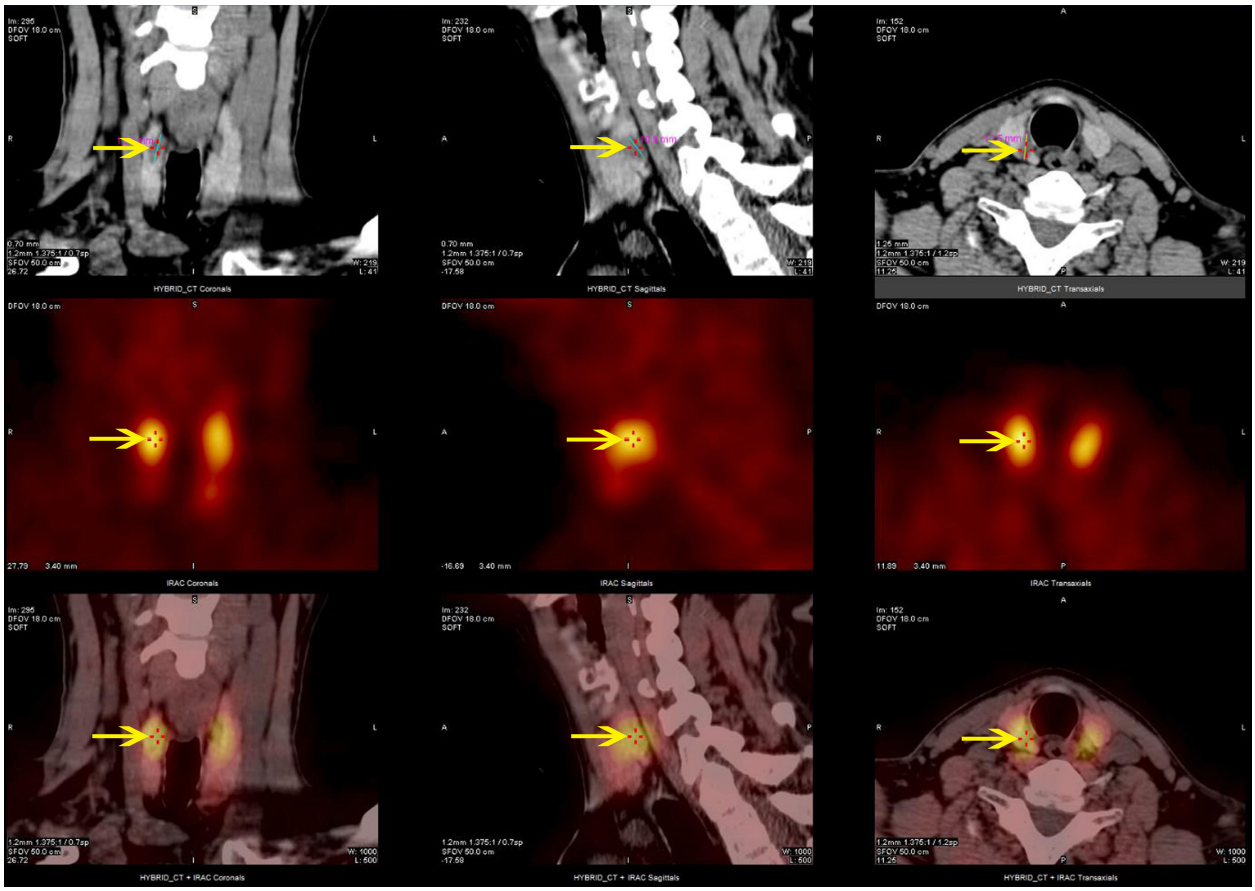

FIGURE 4
Parathyroid SPECT/CT hybrid imaging of the proband's father: nodular parathyroid imaging is observed in the posterior aspect of the lower pole of the right thyroid lobe, with mildly increased MIBI uptake.

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TABLE 1 Results of the proband's and his father's laboratory tests from diagnosis to four weeks postoperatively.

Parameter (reference range and unit)	At the time of Diagnosis (The proband, January, 2019)	Surgery (January 29, 2019)	Six-month postoperative follow-up	Surgery (July 3, 2019)	Four weeks postoperative	At the time of Diagnosis (the proband's father, July, 2019)	Surgery (July 9,2019)	Four weeks postoperative
FBG (3.9-5.9mmol/L)	2.09	Distal pancreatectomy and partial pancreatectomy	4.28-5.02	neck exploration + total parathyroidectomy + partial autotransplantation of the parathyroid gland	NA	5.06	Neck exploration and resection of the right lower parathyroid adenoma	NA
INS (1.5-15μU/ml)	23.96		6.28-13.18		NA	18.80		NA
C-P (0.48-0.78 nmol/L)	0.972				NA	NA		NA
Ca (2.1-2.7mmol/L)	2.69		2.62-2.81		2.33	2.77		2.51
P (0.81-1.45mmol/L)	0.90		1.07-1.33		1.37	0.67		0.95
PTH (1.6-6.9pmol/L)	6.85		9.85-11.35		1.98	24.28		4.2

FBG, fasting blood glucose; INS, Insulin; C-P, C-peptide; Ca, calcium; NA, not available; P, phosphorus; PTH, parathyroid hormone.

within the familial context. Considerable variability in the age of onset, clinical symptoms, disease severity, and types of tumors has previously been documented (12). The presentation of affected glands and their specific pathologies, such as hyperplasia or solitary or multiple parathyroid adenomas, can vary among family members, including identical twins (2, 13). Numerous efforts to analyze clinical characteristics in patients and their relatives with identical variants have verified the absence of straightforward correlations between phenotype and genotype (9, 12, 14, 15). It has been proposed that epigenetic mechanisms activated by environmental factors may affect the phenotype in individuals carrying the identical MEN1 variant (16).The clinical phenotype is therefore heterogeneous both for the variable penetrance of the disease and for the possible influences due to gene-environment interactions (17).

In our case study, whole-exome sequencing was performed on the patient and his parents. But, unfortunately, other family members of the patient were unwilling to undergo genetic testing, and we were unable to construct a detailed pedigree of the disease. We intend to maintain ongoing surveillance of the patient, his offspring, and their family relatives to observe subsequent changes in the MEN1 disease phenotype.

5 Conclusions

To our best understanding, this represented a familial MEN1 attributable to the novel MEN1 variant, c.587delA., which resulted in frameshift translation and abnormal expression of menin. Both the proband and his father were carriers of this identical variant, while exhibited diverse clinical phenotypes and varied symptom severity. Genetic testing is crucial for patients with MEN1 and epigenetic mechanisms may affect the phenotype in individuals carrying the identical variant.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: https://doi.org/10.6084/m9.figshare.27021544.v1.

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (approval number [Ethics (Research) No. 142 of 2024). 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. 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

HH: Formal Analysis, Data curation, Writing – original draft. JL: Writing – review & editing, Resources, Funding acquisition, Formal Analysis, Conceptualization. KZ: Writing – review & editing, Methodology. YT: Writing – review & editing, Data curation. MZ: Writing – review & editing, Conceptualization. ZF: Writing – review & editing, Methodology. TW: Writing – review & editing, Supervision, Investigation, Funding acquisition. YL: Writing – original draft, Visualization, Resources, Investigation.

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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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Conditional associations of sex steroid hormones with C-reactive protein levels in American children and adolescents: evidence from NHANES 2015-2016

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Background: The relationship between sex steroid hormones and high-sensitivity C-reactive protein(hs-CRP) levels in American children and adolescents is understudied. This research will examine this association.

Methods: The study conducted a data analysis from the National Health and Nutrition Examination Survey (NHANES) 2015-2016, adjusting multiple linear regression models with R 4.2.2 and EmpowerStats. A total of 1,768 children and adolescents were surveyed. Data collection involved measurements of serum levels of testosterone, estradiol, sex hormone-binding globulin (SHBG) and hs-CRP.

Results: With the increase in testosterone, a brief rise (β=0.082, *P*=0.047) followed by an overall decline (β=-0.028, *P*=0.023) in hs-CRP was observed in the Male Prepubertal population, while a continuous decline (β=-0.002, *P*<0.05) was seen in the Male Pubertal group. A positive correlation (β=0.047, *P*<0.05) was found between testosterone and hs-CRP in the Female Prepubertal population, whereas no significant association (β=0.002, *P*>0.05) was detected in the Female Pubertal group. A significant inverse correlation was observed between estradiol and hs-CRP solely in the Female Pubertal group (β=-0.002, *P*<0.05), while no association was found in other populations. An inverse relationship between SHBG and hs-CRP was consistently noted across all groups: Male Prepubertal, Male Pubertal, Female Prepubertal, and Female Pubertal.

Conclusions: The association between sex steroid hormones and high-sensitivity C-reactive protein (hs-CRP) levels among American children and adolescents is conditional and influenced by multiple factors.

KEYWORDS

children, adolescents, NHANES, sex steroid hormones, C-reactive protein

Introduction

C-reactive protein (CRP), predominantly produced by hepatocytes in the liver, is a homopentameric protein involved in the acute-phase inflammatory response, and its expression is noticeably heightened during inflammation, such as that caused by rheumatoid arthritis, specific cardiovascular disorders, and infections (1). Estrogens are generally observed to exert an anti-inflammatory influence, potentially enhancing outcomes in severe infections and during wound healing processes (2). However, the literature also indicates that the effects of estrogens on inflammation can vary, being pro-inflammatory, anti-inflammatory, or both, depending on specific cytokines, cell types, and variations in estrogen receptor expression (3). Conversely, testosterone levels are consistently found to have anti-inflammatory effects, as they inhibit adipocyte expansion, differentiation, and function, reduce the production of various cytokines, and promote adiponectin secretion (4). Furthermore, estrogen is speculated to alter CRP concentrations, with evidence indicating a notable impact of hormone replacement therapy (HRT) on CRP levels in the elderly population (1).

Prior research has revealed a complicated and varied relationship between sex steroid hormones and CRP levels in adults. In men, there is generally an inverse association between CRP and key hormonal markers like total testosterone, free testosterone, and SHBG, while the relationship with estradiol is often insignificant (5). Interestingly, estrogen treatment in certain male groups could potentially elevate CRP levels (6). In women, the relationship varies greatly with hormonal and menopausal status, presenting an intricate pattern of positive, negative, or even non-significant correlations between different sex hormones and CRP levels (7–9).

To date, only a single study targeting the adolescent population (aged 12-16 years) has examined the relationship between sex hormones and C-reactive protein. It found a negative correlation between testosterone and high-sensitivity C-reactive protein(hs-CRP) in adolescent boys, but no correlation in girls. In both sexes, a negative association was found between SHBG and hs-CRP (10).

The study of sex steroid hormones in children and adolescents has been attracting increasing scholarly attention (11, 12). Concurrently, existing findings regarding the relationship between these hormones and CRP remain contentious. It is noteworthy that there is a significant scarcity of research, particularly in the age group of 6-11 years. This oversight is notable given the critical developmental changes that occur during these years, which could potentially modulate the influence of sex hormones on inflammation differently than in adults. Understanding these relationships is crucial, as it could enable earlier and more effective interventions for diseases related to inflammation and hormonal imbalances, and transform preventative health strategies. To bridge this research gap, our study proposes to harness the 2015-2016 NHANES data. The objective is to elucidate the potential associations between sex steroid hormones and hs-CRP levels within the population of American children and adolescents. Given the cross-sectional nature of NHANES, our study focuses on identifying patterns and associations rather than causative links, which is a pertinent approach when dealing with observational datasets. Building on this framework, our hypothesis posits that the relationships between sex hormone levels and high-sensitivity C-reactive protein (hs-CRP) vary across different stages of puberty in children and adolescents. We anticipate unique patterns in the correlation between sex hormones and hs-CRP across different genders of children and adolescents before puberty. During puberty, this relationship is expected to mirror the complex interactions observed in adult studies. These findings will provide new insights into the correlations between sex hormones and inflammatory markers during different developmental stages.

Participants and methods

Study design and participants

The National Health and Nutrition Examination Survey (NHANES) is a sequence of cross-sectional studies carried out in the United States, assessing the health and nutritional conditions of both adults and children. In contrast to other research, NHANES integrates physical exams and interviews for comprehensive data gathering. The ethical aspects of each part of the study were reviewed and approved by the National Center for Health Statistics Ethics Review Board, and every participant gave their written informed consent (13). For the purpose of our research, we extracted data from 1,768 children(6-11 years) and adolescents(12-19 years) who had hs-CRP, serum total testosterone (TT), estradiol (E_2), and sex hormone binding globulin (SHBG) records in the 2015-2016 NHANES dataset. The process of our selection is depicted in Figure 1.

Sex hormone indicators as measured in NHANES

The measurement of testosterone, estradiol, and SHBG in NHANES were conducted by NHANES researchers, following standardized procedures.

The acquisition of testosterone and estradiol involves four principal steps: Dissociation of the analytes from binding proteins, extraction of the analytes from the sample matrix, removal of potentially interfering compounds, and quantitation of the analytes by isotope dilution high performance liquid chromatography tandem mass spectrometry (ID-LC-MS/MS) using stable isotope labeled internal standards and external calibrators. Isolation of the analytes is achieved using liquid-liquid extraction. ID-LC-MS/MS is performed with a triple quadrupole mass spectrometer using electrospray ionization in positive ion mode for testosterone, and negative ion mode for estradiol. Estradiol and testosterone are identified based on chromatographic retention time and on specific mass to charge ratio transitions using selected reaction monitoring (SRM). A ^{13}C isotope-labeled testosterone and a ^{13}C isotope-labeled estradiol are used as internal standards.

The method for measuring Sex Hormone Binding Globulin (SHBG) is based on the reaction of SHBG with immuno-antibodies and chemo-luminescence measurements of the reaction products. It

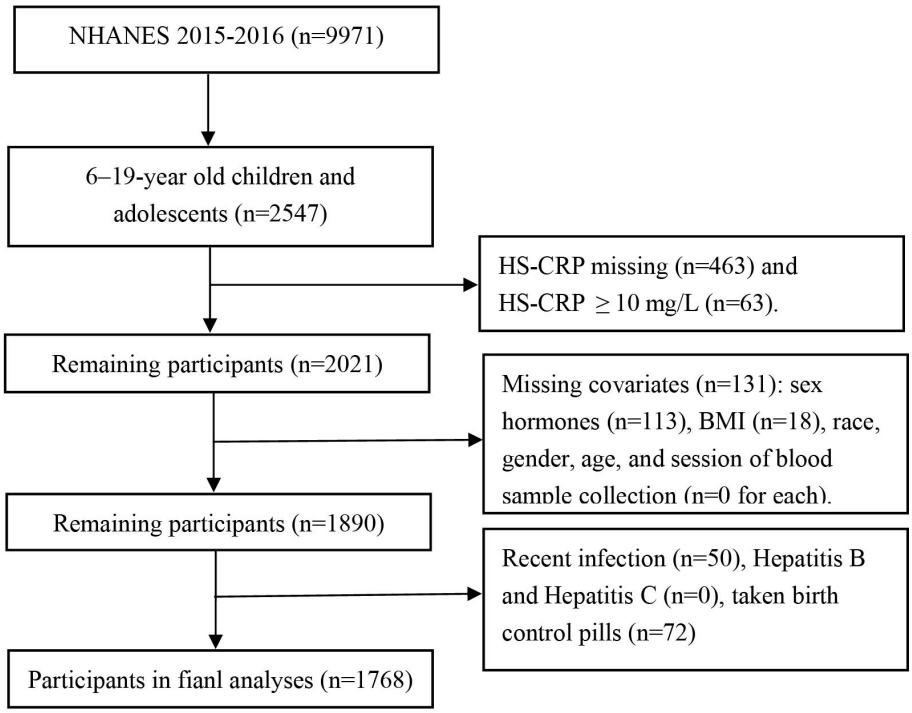

FIGURE 1
Flowchart for selecting the study population. NHANES, The National Health and Nutrition Examination Survey.

consists of 2 incubation steps and a chemiluminescent measurement via photomultiplier tube that spans 18 minutes.The first incubation period begins by sandwiching the sample of SHBG containing serum between a biotinylated monoclonal SHBG-specific antibody and a monoclonal SHBG-specific antibody that is labeled with ruthenium. The second incubation entails the addition of streptavidin-coated microparticles to the sample mixture. The microparticles bind to the solid phase via biotin and streptavidin interactions. The resulting sample mixture is then aspirated into a measuring cell that is subjected to a magnetic field. This captures the microparticles on an electrode. The remains of the sample mixture are subsequently washed out of the measuring cell. A voltage is applied to the electrode causing a chemiluminescent reaction that is measured by a photomultiplier tube. The readings are compared to an instrument- and lot-specific calibration curve.

High-sensitivity C-reactive protein as measured in NHANES

High-sensitivity C-reactive protein (hs-CRP) levels were obtained from the NHANES 2015-2016 dataset, where the hs-CRP measurements were carried out by NHANES staff using a dual-agent immunoturbidimetric system at designated laboratories. Initially, a sample is amalgamated with a Tris buffer followed by an incubation period. Then, the second reagent, comprising latex particles that are coated with mouse anti-human CRP antibodies, is introduced. Upon exposure to circulating CRP, the latex particles tend to clump together, leading to the formation of immune complexes. These formed complexes induce an increase in the scattering of light, which is directly proportional to the CRP concentration. The resulting light absorption from the scattered light is then measured against a pre-established CRP standard curve to determine the concentration of CRP (14).

Covariates

The selection of covariates in this study was based on previous research on sex hormones (5, 8, 9), and included age (continuous), race (Mexican American, other Hispanic, Non-Hispanic white, Non-Hispanic black, Non-Hispanic Asian, or other race), education level (6th and below 6th grade, above 6th grade), poverty income ratio (PIR, continuous), body mass index (BMI, continuous), diabetes (categorical), session of blood sample collection (morning, afternoon, or evening), total cholesterol(TC, continuous), and physical activity(non-activity, 0.1-0.9 hour/week, 0.1-0.9 hour/week, 1.0-3.4 hour/week, 3.5-5.9 hour/week, and ≥6 hour/week). To identify participants with diabetes, any of the following characteristics were used: (a) hemoglobin A1C concentration ≥ 6.5% or a fasting plasma glucose level ≥126 mg/dL; (b) for those who answered "yes" to the following questions:

'Take diabetic pills to lower blood sugar?' or 'Doctor told you have diabetes?' or 'Taking insulin now?' (15).

Adjustment for pubertal status

It is noteworthy that segmenting the participants aged 6-19 years into categories of children and adolescents based purely on age could result in blending prepubescent and pubescent individuals within each group. Consequently, this could give rise to unusually high or low concentrations of sex hormones within each category, which could skew the relationships between sex hormones and hs-CRP during regression analysis. In addition, the influence of sex hormones on hs-CRP may vary with puberty status. In an effort to mitigate this concern, we subdivided the participants into two categories, namely pubertal and prepubertal groups, based on their serum sex hormone levels and menarcheal status. Participants who displayed levels of TT ≥ 50 ng/dL (in the case of males) or E_2 ≥ 20 pg/mL (in the case of females), or had initiated menstrual cycles (applicable to females), were classified under the 'pubertal group'. The remaining participants, who did not meet these criteria, were assigned to the 'prepubertal group' (16).

Statistical analyses

We used sampling weights to adjust for selection probabilities, oversampling, non-response, and differences between the sample and the entire US population. Given the right-skewed distribution of hs-CRP, we performed a natural logarithmic transformation on the hs-CRP values.Weighted univariate linear regression was employed to ascertain the correlations between sex steroid hormones and hs-CRP. We used weighted multivariable linear regression models to examine the relationship between sex hormones and hs-CRP, adjusting for various confounding factors. We performed trend tests to see if the effects of different ranges of sex hormones on hs-CRP were consistent. We adopted the Generalized Additive Model(GAM) to detect the non-linear association. If the relationship between sex hormones and hs-CRP was non-linear, a two-piecewise linear regression model was implemented to estimate the threshold effect of sex steroid hormones on hs-CRP. Subgroup analyses were conducted via stratified linear regression models, with the modification and interaction within subgroups assessed through the likelihood ratio test. These models were designed to explore associations between sex hormones and hs-CRP levels, rather than to infer causal relationships. Given the cross-sectional nature of the NHANES data, the causal direction of any observed associations cannot be determined. Therefore, we selected an analytical approach ideal for identifying correlations in cross-sectional data. We analyzed the data using the R software (version 4.2.2) and EmpowerStats (http://www.empowerstats.com). We considered a *P*-value < 0.05 as statistically significant.

Results

Baseline characteristics

The study population (Table 1), stratified by gender and age groups, exhibited significant disparities in levels of testosterone, estradiol, SHBG, age, and BMI (all $P<0.001$). Testosterone and estradiol levels were notably higher in adolescents, while SHBG was highest in children. BMI differed significantly between children and adolescents. Most participants were of normal weight (58.26%) and non-diabetic (99.38%).Importantly, pubertal status significantly differed between children and adolescents ($P<0.001$), with a noteworthy proportion of female children (21.85%) already in puberty. This finding prompted us to mainly categorize our population based on pubertal status for subsequent analysis.

Weighted univariate analysis

The Supplementary Table 1 analysis revealed that testosterone, estradiol, SHBG, age, race, BMI, diabetes status, and examination time were significantly associated with hs-CRP levels across prepubertal and pubertal males and females. Notably, the association strength did not significantly differ between groups, barring estradiol, race, examination time, and BMI.

Association between testosterone and high-sensitivity C-reactive protein

In Male Prepubertal subjects, testosterone exhibited no significant link with hs-CRP (Table 2). A threshold effect was evident at a testosterone level of 8.90 ng/dl (Supplementary Table 2), associating positively below(β=0.082, $P=0.047$) and negatively above(β=-0.028, $P=0.023$) this threshold (Figure 2). Stratified analysis (Supplementary Table 3) revealed significant negative correlations in Mexican American(β=-0.027, $P<0.05$) and Other Hispanic groups(β=-0.044, $P<0.05$), while the Other Race group showed a positive correlation(β=0.073, $P<0.05$).

In Male Pubertal subjects, a consistent negative association was evident between testosterone and hs-CRP levels (β=-0.002, P<0.05, Table 2). Supplementary Table 2 revealed a threshold effect at 224.00 ng/dl, where above this level, the association was more pronounced(β=-0.003, P<0.001) (Figure 2). The results of the stratified analysis (Supplementary Table 3) also support the negative correlation between testosterone and hs-CRP.

For Female Prepubertal subjects, a significant positive association was found between testosterone and hs-CRP in the fully-adjusted model in Table 2 (β=0.047, $P<0.05$). However, the piecewise linear regression model in Supplementary Table 2 failed to detect any significant threshold effect (Supplementary Figure 1). In the stratified analysis for the Female Prepubertal group (Supplementary Table 3), a significant negative relationship is demonstrated in overweight children (β=-0.077, $P<0.05$).

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TABLE 1 Characteristics of the population.

Characteristic	Total population	Total population [a]	Male Children (6–11 years)	Male Adolescents (12–19 years)	Female Children (6–11 years)	Female Adolescents (12–19 years)	P-value
Testosterone (ng/dl, median, Q1-Q3)	18.25 (4.26-183.25)	24.00 (5.22-256.00)	3.62 (2.11-5.89)	387.00 (249.75-549.00)	4.62 (2.86-9.55)	24.50 (17.80-32.20)	<0.001
Estradiol (pg/ml, median,Q1-Q3)	12.35 (2.11-31.55)	15.00 (2.11-33.70)	2.11 (2.11-2.11)	19.40 (11.80-26.82)	2.11 (2.11-13.90)	57.10 (34.20-116.00)	<0.001
SHBG (nmol/l, median, Q1-Q3)	56.76 (34.41-95.55)	57.25 (34.77-93.93)	94.95 (62.72-134.88)	33.83 (22.85-47.23)	80.81 (49.66-112.70)	48.66 (33.41-74.41)	<0.001
Hs-CRP (mg/L, median, Q1-Q3)	0.40 (0.08-1.20)	0.30 (0.08-1.10)	0.30 (0.08-1.20)	0.40 (0.08-1.17)	0.30 (0.08-1.10)	0.40 (0.08-1.40)	0.668
Age (year, mean ± SD)	12.00 ± 3.86	12.48 ± 0.10	8.56 ± 1.69	15.35 ± 2.23	8.53 ± 1.72	14.98 ± 2.13	<0.001
PIR (median, Q1-Q3)	1.47 (0.65-2.75)	2.04 (0.98-3.52)	1.52 (0.73-2.99)	1.42 (0.61-2.68)	1.50 (0.58-2.67)	1.47 (0.74-2.64)	0.498
BMI (kg/m^2, mean ± SD)	21.63 ± 5.80	21.77 ± 0.28	18.84 ± 4.06	24.00 ± 6.25	18.99 ± 4.11	24.33 ± 5.71	<0.001
BMI Category [b]							0.150
Underweight (N,%)	45 (2.55%)	3.30%	8 (1.87%)	20 (3.89%)	10 (2.38%)	7 (1.73%)	
Normal Weight (N,%)	1030 (58.26%)	58.79%	257 (60.05%)	294 (57.20%)	257 (61.05%)	222 (54.81%)	
Overweight (N,%)	319 (18.04%)	18.05%	67 (15.65%)	89 (17.32%)	76 (18.05%)	87 (21.48%)	
Obese (N,%)	374 (21.15%)	19.86%	96 (22.43%)	111 (21.60%)	78 (18.53%)	89 (21.98%)	
TC (mg/dL, mean ± SD)	154.98 (27.63)	154.77 (0.95)	157.43 (25.20)	152.20 (28.49)	156.36 (27.08)	154.46 (29.29)	0.021
Race							0.438
Mexican American (N,%)	432 (24.43%)	16.68%	99 (23.13%)	119 (23.15%)	111 (26.37%)	103 (25.43%)	
Other Hispanic (N,%)	248 (14.03%)	9.86%	65 (15.19%)	56 (10.89%)	66 (15.68%)	61 (15.06%)	
Non-Hispanic White (N,%)	445 (25.17%)	50.26%	104 (24.30%)	145 (28.21%)	103 (24.47%)	93 (22.96%)	
Non-Hispanic Black (N,%)	367 (20.76%)	12.80%	92 (21.50%)	112 (21.79%)	83 (19.71%)	80 (19.75%)	
Non-Hispanic Asian (N,%)	168 (9.50%)	5.02%	43 (10.05%)	54 (10.51%)	29 (6.89%)	42 (10.37%)	
Other Race (N,%)	108 (6.11%)	5.38%	25 (5.84%)	28 (5.45%)	29 (6.89%)	26 (6.42%)	
Education							<0.001
6th and below 6th grade (N,%)	1005 (56.84%)	50.99%	428 (100.00%)	79 (15.37%)	421 (100.00%)	77 (19.01%)	
Above 6th grade (N,%)	763 (43.16%)	49.01%	0 (0.00%)	435 (84.63%)	0 (0.00%)	328 (80.99%)	
Diabetes							0.753
Non-diabetes (N,%)	1757 (99.38%)	99.41%	426 (99.53%)	511 (99.42%)	419 (99.52%)	401 (99.01%)	
Diabetes (N,%)	11 (0.62%)	0.59%	2 (0.47%)	3 (0.58%)	2 (0.48%)	4 (0.99%)	
Session of blood sample collection							0.004
Morning (N,%)	738 (41.74%)	41.69%	154 (35.98%)	238 (46.30%)	161 (38.24%)	185 (45.68%)	
Afternoon (N,%)	663 (37.50%)	37.55%	168 (39.25%)	190 (36.96%)	162 (38.48%)	143 (35.31%)	

(Continued)

TABLE 1 Continued

Characteristic	Total population	Total population [a]	Male Children (6–11 years)	Male Adolescents (12–19 years)	Female Children (6–11 years)	Female Adolescents (12–19 years)	P-value
Evening (N,%)	367 (20.76%)	20.76%	106 (24.77%)	86 (16.73%)	98 (23.28%)	77 (19.01%)	
Physical activity (hour / week) [c]							<0.001
Non-activity (N,%)	1385 (78.34%)	71.92%	428 (100.00%)	264 (51.36%)	421 (100.00%)	272 (67.16%)	
0.1-0.9 (N,%)	45 (2.55%)	2.99%	0 (0.00%)	29 (5.64%)	0 (0.00%)	16 (3.95%)	
1.0-3.4 (N,%)	122 (6.90%)	9.44%	0 (0.00%)	65 (12.65%)	0 (0.00%)	57 (14.07%)	
3.5-5.9 (N,%)	58 (3.28%)	4.54%	0 (0.00%)	41 (7.98%)	0 (0.00%)	17 (4.20%)	
≥6 (N,%)	158 (8.94%)	11.12%	0 (0.00%)	115 (22.37%)	0 (0.00%)	43 (10.62%)	
Puberty Status [d]							<0.001
Pubertal (N,%)	1000 (56.56%)	61.84%	30 (7.01%)	482 (93.77%)	92 (21.85%)	396 (97.78%)	
Prepubertal (N,%)	768 (43.44%)	38.16%	398 (92.99%)	32 (6.23%)	329 (78.15%)	9 (2.22%)	

[a]Applied sampling weights. In this column, values following the mean denote Standard Error (SE), not Standard Deviation (SD);
[b]Underweight (BMI < 5th percentile), Normal weight (BMI 5th to < 85th percentiles), Overweight (BMI 85th to < 95th percentiles), Obese (BMI ≥ 95th percentile);
[c]Physical activity pertains to individuals aged 12 and above;
[d]Puberty status was defined as "pubertal" if testosterone ≥ 50 ng/dL in males, estradiol ≥ 20 pg/ml or menstrual period started in females, otherwise puberty status was defined as "prepubertal";
SHBG, sex hormone-binding globulin; Hs-CRP, High-Sensitivity C-Reactive Protein; PIR, Poverty income ratio; BMI, body mass index; TC, Total Cholesterol

For Female Pubertal subjects, no significant association between testosterone and CRP was found in all models in Table 2, and likewise, no significant threshold effect was detected in Supplementary Table 2 and Supplementary Figure 1. In the stratified analysis of female pubertal development, a significant negative association was observed among Mexican American children(β=-0.016, P<0.05) and those engaging in over 6 hours of physical activity per week(β=-0.048, P<0.05). Conversely, a significant positive relationship was found in Non-Hispanic Asian children (β=0.023, P<0.05).

Association between estradiol and high-sensitivity C-reactive protein

In conducting multivariate regression analysis (Table 2) and threshold effect analysis (Supplementary Table 2), we did not identify significant associations between estradiol and hs-CRP in Male Prepubertal, Male Pubertal, and Female Prepubertal cohorts (Figure 2; Supplementary Figure 1). However, upon conducting stratified analyses within these three populations, we identified several subgroups where the relationship between estradiol and hs-CRP exhibited statistically significant associations. These included both positive and negative correlations (Supplementary Table 4).

In the Female Pubertal group, a significant negative correlation was observed between estradiol and hs-CRP(β=-0.002, P<0.05), and this inverse relationship was more pronounced when estradiol was less than or equal to 183 pg/ml(β=-0.004, P=0.001). In the Female Pubertal group, a significant positive correlation was observed between estradiol and hs-CRP when physical activity was between 3.5-5.9 hours per week(β=0.017, P<0.05, Supplementary Table 4).

Association between SHBG and high-sensitivity C-reactive protein

From Table 2, for each subgroup, there exists a significant negative association between SHBG and hs-CRP, with the relationship holding across various models adjusting for different sets of covariates(all P<0.05). For the Male Prepubertal, Male Pubertal, Female Prepubertal, and Female Pubertal groups, the β coefficients suggest a stronger negative correlation below the respective inflection points of 72.09, 25.74, 149.10, and 56.48 nmol/l (Supplementary Table 2, Figure 2, and Supplementary Figure 1).

The results from stratified analysis also support a negative correlation between SHBG levels and hs-CRP (Supplementary Table 5). However, a notable exception is observed in the 'Above 6th grade' group within the Female Prepubertal population, where a positive correlation is identified(β=0.035, P<0.05).

Discussion

Our research uncovers a distinct gender and age-related difference in the relationship between testosterone and hs-CRP. In Male Prepubertal group, an initial increase in testosterone levels leads to a transient surge in hs-CRP (β=0.082, P=0.047), subsequently resulting in an overall decline (β=-0.028, P=0.023). Contrarily, in Male Pubertal, hs-CRP levels consistently decrease under the influence of testosterone (β=-0.002, P<0.05). In Female Prepubertal, a significant positive correlation between testosterone and hs-CRP is observed (β=0.047, P<0.05). However, no significant correlation is found in Female Pubertal (β=0.002, P>0.05). Previous studies commonly support the negative correlation between testosterone

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TABLE 2 Association between sex steroid hormones and high-sensitivity C-reactive protein.

Sex steroid hormones	Non-adjusted model β, 95%CI	Minimally-adjusted model β, 95%CI	Fully-adjusted model β, 95%CI
Testosterone (Male Prepubertal)	0.011 (-0.006, 0.027)	-0.012 (-0.032, 0.008)	-0.012 (-0.033, 0.008)
Q1(0.53 -4.24 ng/dl)	Reference	Reference	Reference
Q2(4.27 -18.20 ng/dl)	0.277 (-0.031, 0.584)	-0.002 (-0.355, 0.351)	-0.006 (-0.363, 0.350)
Q3(18.30 -181.00 ng/dl)	0.270 (-0.273, 0.812)	-0.372 (-1.014, 0.271)	-0.392 (-1.045, 0.261)
Q4(190.00 -1140.00 ng/dl)	0.236 (-0.268, 0.622)	-0.492 (-1.312, 0.618)	-0.218 (-1.362, 0.136)
P for trend	0.270	0.188	0.237
Testosterone (Male Pubertal)	-0.001 (-0.002, -0.000)	-0.002 (-0.002, -0.001)	-0.002 (-0.003, -0.001)
Q1(0.53 -4.24 ng/dl)	Reference	Reference	Reference
Q2(4.27 -18.20 ng/dl)	-0.000 (-0.027, 0.027)	-0.002 (-0.128, -0.001)	-0.016 (-0.032, -0.002)
Q3(18.30 -181.00 ng/dl)	-0.009 (-0.011, 0.030)	-0.017 (-0.006, 0.039)	-0.022 (-0.062, 0.008)
Q4(190.00 -1140.00 ng/dl)	0.193 (-0.159, 0.544)	-0.004 (-0.388, 0.379)	-0.051 (-0.456, 0.354)
P for trend	0.028	0.024	<0.001
Testosterone (Female Prepubertal)	0.055 (0.016, 0.093)	0.045 (-0.000, 0.091)	0.047 (0.001, 0.093)
Q1(0.53 -4.24 ng/dl)	Reference	Reference	Reference
Q2(4.27 -18.20 ng/dl)	0.303 (-0.017, 0.624)	0.118 (-0.244, 0.480)	0.153 (-0.213, 0.520)
Q3(18.30 -181.00 ng/dl)	1.140 (0.033, 2.247)	1.084 (-0.092, 2.260)	1.010 (-0.184, 2.203)
Q4(190.00 -1140.00 ng/dl)	0.392 (-0.483, 0.461)	0.739 (-0.830, 0.949)	0.715 (-0.039, 2.491)
P for trend	<0.001	0.061	0.046
Testosterone (Female Pubertal)	0.008 (-0.002, 0.017)	0.004 (-0.005, 0.014)	0.002 (-0.007, 0.012)
Q1(0.53 -4.24 ng/dl)	Reference	Reference	Reference
Q2(4.27 -18.20 ng/dl)	1.519 (-1.297, 4.335)	2.056 (-0.682, 4.794)	1.792 (-0.916, 4.500)
Q3(18.30 -181.00 ng/dl)	1.495 (-1.317, 4.306)	1.948 (-0.777, 4.674)	1.672 (-1.027, 4.370)
Q4(190.00 -1140.00 ng/dl)	0.986 (-0.110, 3.062)	1.015 (-0.609, 3.039)	1.017 (-0.036, 3.070)
P for trend	0.312	0.171	0.659
Estradiol (Male Prepubertal)	0.261 (0.050, 0.472)	0.183 (-0.028, 0.395)	0.185 (-0.031, 0.401)
Q1(2.11-6.18 pg/ml)	Reference	Reference	Reference
Q2(6.20-12.30 pg/ml)	0.742 (-0.163, 1.648)	0.430 (-0.471, 1.330)	0.405 (-0.510, 1.320)
Q3(12.40-31.50 pg/ml)	0.167 (-0.189, 0.185)	0.178 (-0.205, 1.151)	0.175 (-0.232,1.117)
Q4(31.70-564.00 pg/ml)	-0.298 (-0.319, 0.276)	-0.310 (-0.333, 0.288)	-0.300 (-0.353, 0.247)
P for trend	0. 012	0.350	0.094
Estradiol (Male Pubertal)	0.016 (0.005, 0.028)	0.013 (-0.002, 0.027)	0.012 (-0.004, 0.027)
Q1(2.11-6.18 pg/ml)	Reference	Reference	Reference
Q2(6.20-12.30 pg/ml)	0.791 (-0.234, 1.349)	0.738 (-0.161, 1.315)	0.729 (-0.132, 1.326)
Q3(12.40-31.50 pg/ml)	0.991 (-0.431, 1.551)	0.923 (-0.294, 1.551)	0.936 (-0.279, 1.594)
Q4(31.70-564.00 pg/ml)	1.274 (-0.539, 2.010)	1.208 (-0.393, 2.024)	1.235 (-0.390, 2.080)
P for trend	0.001	0.088	0.089
Estradiol (Female Prepubertal)	0.037 (-0.005, 0.079)	0.007 (-0.041, 0.055)	0.016 (-0.033, 0.065)
Q1(2.11-6.18 pg/ml)	Reference	Reference	Reference

(Continued)

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TABLE 2 Continued

Sex steroid hormones	Non-adjusted model β, 95%CI	Minimally-adjusted model β, 95%CI	Fully-adjusted model β, 95%CI
Q2(6.20-12.30 pg/ml)	0.284 (-0.093, 0.660)	-0.026 (-0.458, 0.406)	0.037 (-0.401, 0.476)
Q3(12.40-31.50 pg/ml)	0.167 (-0.189, 0.145)	-0.178 (-0.205, 0.151)	-0.175 (-0.232, 0.117)
Q4(31.70-564.00 pg/ml)	0.298 (-0.319, 0.276)	-0.310 (-0.333, 0.288)	-0.300 (-0.353, 0.247)
P for trend	0.141	0.907	0.867
Estradiol (Female Pubertal)	-0.002 (-0.003, -0.000)	-0.003 (-0.004, -0.001)	-0.002 (-0.004, -0.001)
Q1(2.11-6.18 pg/ml)	Reference	Reference	Reference
Q2(6.20-12.30 pg/ml)	-0.094 (-0.119, 0.069)	-0.101 (-0.129, 0.074)	-0.099 (-0.160, 0.037)
Q3(12.40-31.50 pg/ml)	-0.142 (-0.734, 0.450)	0.000 (-0.573, 0.574)	-0.054 (-0.619, 0.512)
Q4(31.70-564.00 pg/ml)	-0.255 (-0.817, 0.307)	-0.207 (-0.750, 0.337)	-0.297 (-0.837, 0.243)
P for trend	0.027	0.034	0.033
SHBG(Male Prepubertal)	-0.016 (-0.019, -0.013)	-0.015 (-0.018, -0.012)	-0.015 (-0.018, -0.012)
Q1(4.81-34.31 nmol/l)	Reference	Reference	Reference
Q2(34.44-56.51 nmol/l)	-0.654 (-1.226, -0.082)	-0.627 (-1.200, -0.054)	-0.611 (-1.190, -0.032)
Q3(56.76-95.54 nmol/l)	-1.776 (-2.285, -1.266)	-1.697 (-2.222, -1.172)	-1.707 (-2.240, -1.175)
Q4(95.57-293.20 nmol/l)	-2.475 (-2.962, -1.988)	-2.365 (-2.879, -1.850)	-2.393 (-2.916, -1.870)
P for trend	<0.001	0.013	0.013
SHBG(Male Pubertal)	-0.021 (-0.026, -0.016)	-0.022 (-0.028, -0.017)	-0.023 (-0.029, -0.017)
Q1(4.81-34.31 nmol/l)	Reference	Reference	Reference
Q2(34.44-56.51 nmol/l)	-0.448 (-0.707, -0.189)	-0.439 (-0.707, -0.171)	-0.438 (-0.705, -0.170)
Q3(56.76-95.54 nmol/l)	-0.974 (-1.335, -0.613)	-0.970 (-1.363, -0.577)	-0.943 (-1.341, -0.544)
Q4(95.57-293.20 nmol/l)	-1.576 (-2.252, -0.901)	-1.609 (-2.323, -0.895)	-1.669 (-2.391, -0.947)
P for trend	<0.001	0.041	0.020
SHBG(Female Prepubertal)	-0.015 (-0.018, -0.012)	-0.014 (-0.017, -0.011)	-0.014 (-0.018, -0.011)
Q1(4.81-34.31 nmol/l)	Reference	Reference	Reference
Q2(34.44-56.51 nmol/l)	-0.477 (-1.075, 0.120)	-0.410 (-1.016, 0.195)	-0.445 (-1.057, 0.168)
Q3(56.76-95.54 nmol/l)	-1.334 (-1.898, -0.769)	-1.210 (-1.786, -0.634)	-1.213 (-1.797, -0.629)
Q4(95.57-293.20 nmol/l)	-1.929 (-2.474, -1.384)	-1.792 (-2.359, -1.225)	-1.823 (-2.395, -1.251)
P for trend	<0.001	0.011	<0.001
SHBG(Female Pubertal)	-0.013 (-0.017, -0.010)	-0.013 (-0.016, -0.010)	-0.013 (-0.016, -0.009)
Q1(4.81-34.31 nmol/l)	Reference	Reference	Reference
Q2(34.44-56.51 nmol/l)	-0.763 (-1.080, -0.446)	-0.672 (-0.982, -0.362)	-0.551 (-0.863, -0.239)
Q3(56.76-95.54 nmol/l)	-1.175 (-1.494, -0.857)	-1.095 (-1.408, -0.782)	-1.029 (-1.340, -0.717)
Q4(95.57-293.20 nmol/l)	-1.559 (-1.974, -1.144)	-1.472 (-1.885, -1.060)	-1.434 (-1.844, -1.025)
P for trend	<0.001	0.019	0.021

SHBG, sex hormone-binding globulin; The number of participants in each quartile group (Q1-Q4) is 442 individuals;
Non-adjusted model: no covariates were adjusted; Minimally-adjusted model: adjusted for age and race;
Fully-adjusted model: adjusted for age, race, education level, poverty income ratio, diabetes status, session of blood sample collection, total cholesterol. All models were weighted.

and CRP in both adolescent (10) and adult males (5, 17), to some extent, validating the hypothesis that testosterone has anti-inflammatory properties in males. In females, the relationship between testosterone and CRP appears diverse, even contrary. The research conducted by de Dios O and colleagues did not identify any correlation between testosterone and hs-CRP in female adolescents aged between 12 and 16 years (10). In postmenopausal women, some studies have reported a positive correlation between serum

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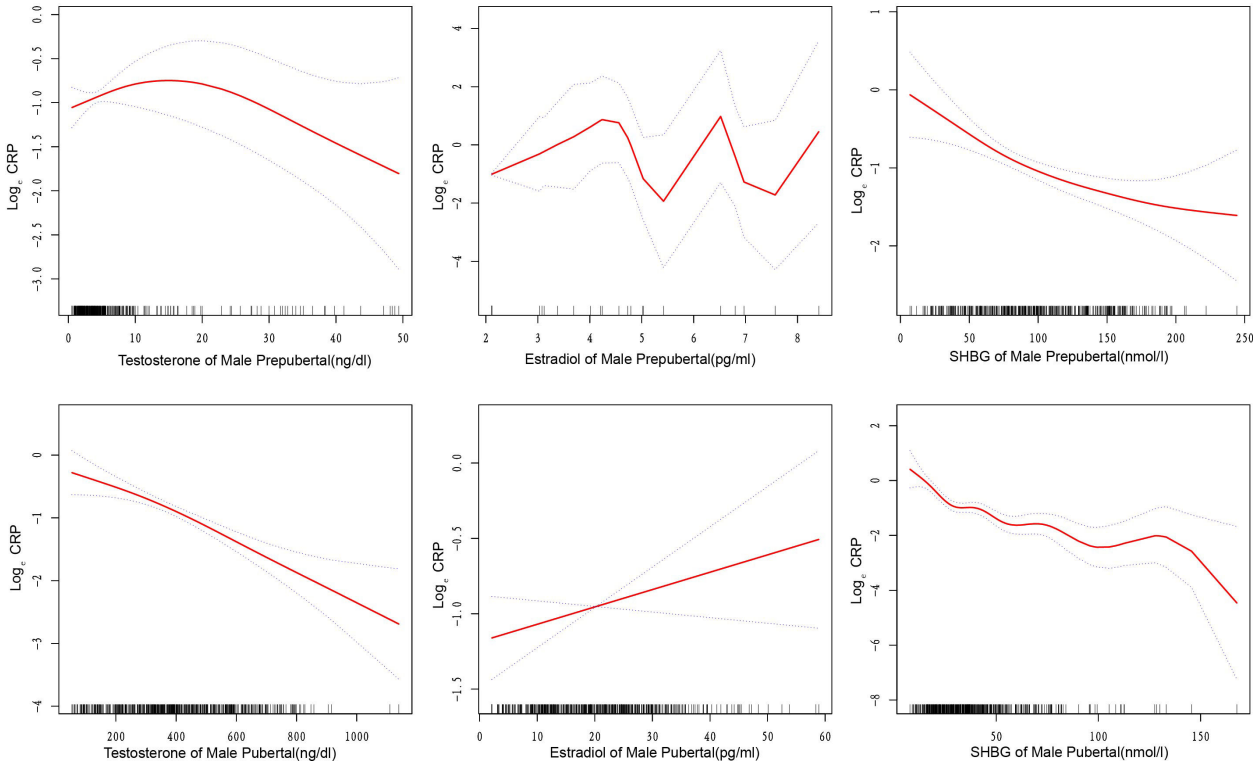

FIGURE 2
Curve fitting relationships between sex steroid hormones and high-sensitivity C-reactive protein in male children and adolescents. The solid and dashed lines in the graph represent the estimated values and corresponding 95% confidence intervals of high-sensitivity C-reactive protein, respectively. SHBG, sex hormone-binding globulin; Curve adjusted for age, race, education level, poverty income ratio, diabetes status, sample collection session, and total cholesterol.

testosterone levels and CRP (8). However, there are also studies concluding an inverse relationship between serum testosterone levels and CRP in postmenopausal women (9, 18). The level of testosterone plays a crucial role in modulating inflammatory processes, which is achieved by suppressing the expansion, differentiation, and function of adipocytes, curtailing the formation of cytokines (leptin, IL-6, TNF-α, MCP-1, resistin), and concurrently promoting the secretion of adiponectin (4). Future research is necessary to explore the interaction mechanisms between testosterone and hs-CRP.

Our study found a negative correlation between estradiol and hs-CRP (β=-0.002, P<0.05) solely within the Female Pubertal population. No such correlation was observed in the Male Prepubertal, Male Pubertal, and Female Prepubertal groups. Previous studies on adult females have indicated that the relationship between estradiol and CRP is negative pre-menopause (7, 19), but turns positive post-menopause (8, 18). This suggests a potentially complex and dynamic relationship between these variables across different stages of life. Throughout the inflammatory reaction, pro-inflammatory cytokines prompt the synthesis of NO in cells like monocytes, macrophages, and neutrophils. During the process of phagocytosis, NO, when discharged by tissue macrophages, operates as a positive feedback entity, catalyzing the attraction of additional phagocytes. Physiological levels of estrogen maintained the nCRP-facilitated decrease in NO production in LPS-stimulated monocytes, concurrently, estrogen countered the mCRP-induced increase of NO production in the same cells (20). This aligns with earlier research demonstrating that estrogen attenuates pro-inflammatory responses, which includes the production of NO and inflammatory cytokines.

Our study reveals a consistent negative correlation between SHBG and hs-CRP in both children and adolescents. This finding is not isolated, as it aligns with the results of prior research. Such negative correlation between SHBG and C-reactive protein has been confirmed in various populations, including adolescents (10), adult males (5), premenopausal women (19), and postmenopausal women (9). SHBG, secreted by the liver into the bloodstream, avidly binds to both androgens and estrogens, thereby regulating their bioavailability. BMI has been traditionally considered a primary determinant of circulating SHBG concentration, with a reported consistent negative correlation between BMI and plasma SHBG levels (21). Lower serum SHBG concentrations in overweight individuals serve as a biomarker of metabolic syndrome (22) and indicate an increased risk of type 2 diabetes(T2D) (23) and cardiovascular diseases(CVD) (24). Obesity-related endocrine mechanisms and chronic inflammation are associated with the

reduction of SHBG prior to puberty, suggesting that lower SHBG levels could indicate an earlier onset of puberty (25). Reviews have proposed that the subtle inflammation and changes in pro-inflammatory/anti-inflammatory cytokines (i.e., TNFα, IL-1β, and adiponectin) occurring in obesity and T2D may be the primary cause of decreased SHBG levels, rather than hyperinsulinemia (24). It remains to be elucidated whether the decrease in SHBG is merely a biomarker, or if it actively participates in the inflammatory response process related to CRP, and subsequently contributes to the pathogenesis of obesity, T2D, fatty liver, and cardiovascular diseases.

Our study possesses several strengths. First, we have filled a research gap in exploring the relationship between sex steroid hormones and hs-CRP in children populations(6-11 years). Additionally, we categorized children and adolescents into prepubertal and pubertal groups based on hormone levels, offering a more reliable demographic foundation for researching the association between sex steroid hormones and hs-CRP. Furthermore, the data utilized in our study was obtained from the NHANES database, ensuring the accuracy of serum hormone and hs-CRP measurements. We also weighted our data to ensure that our final results would be representative of the overall health status of children and adolescents in the United States.

Nevertheless, there are some limitations in our study. Although we conducted detailed stratified analyses for various covariates, the unequal distribution of sample sizes across certain groups may have resulted in insufficient statistical power for some analyses. For instance, in the stratified analysis of testosterone and high-sensitivity C-reactive protein (Supplementary Table 3), the Normal Weight group included 1,030 participants, while the Underweight group comprised only 45 participants. Therefore, the interpretation of these results must take into account the limitations posed by the small sample sizes. Even though we adjusted for potential confounders, there might still be unadjusted factors that could influence our results. The lack of data on gonadotropin-releasing hormone, gonadotropins, and crucial enzymes involved in hormone responses restricted us from further delving into the underlying biological mechanisms. Importantly, it should be noted that our study is cross-sectional, thereby preventing us from establishing a causal relationship between sex steroid hormones and hs-CRP. This design captures data at a single point in time, limiting our ability to track changes in variables over time or to ascertain causal sequences. Future research should employ longitudinal designs to overcome these limitations and validate our findings.

Conclusions

Our findings indicate that the association between sex steroid hormones and high-sensitivity C-reactive protein (hs-CRP) levels among American children and adolescents is conditional and influenced by multiple factors. Specifically, this association is influenced by several factors including age, body mass index (BMI), pubertal status, hormone levels, among others. For instance, in prepubertal males, testosterone levels below 8.90 ng/dL positively correlate with hs-CRP levels, while levels above this threshold show a negative correlation. This indicates that the association between sex steroid hormones and inflammatory markers involves a multifaceted interplay, potentially including other biomarkers and environmental factors not assessed in this study. Further research is needed to explore these dimensions to better elucidate the correlations between sex hormones and inflammatory markers.

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 humans were approved by NCHS Ethics Review Board (ERB) Approval. 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. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

ZSZ: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. XGL: Conceptualization, Resources, Writing – review & editing, Methodology, Project administration. CYW: Conceptualization, Formal analysis, Funding acquisition, Methodology, Writing – review & editing. SZZ: Data curation, Funding acquisition, Investigation, Validation, Visualization, Writing – review & editing. XYZ: Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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www.researchsquare.com/article/rs-3935965/v1) (26), which has not undergone peer review.

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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1431984/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Curve fitting relationships between sex steroid hormones and high-sensitivity C-reactive protein in female children and adolescents. The solid and dashed lines in the graph represent the estimated values and corresponding 95% confidence intervals of high-sensitivity C-reactive protein, respectively. SHBG: sex hormone-binding globulin; Curve adjusted for age, race, education level, poverty income ratio, diabetes status, sample collection session, and total cholesterol.

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Self-puberty staging in endocrine encounters during the COVID pandemic

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Background: Evaluation of pubertal development is crucial in Endocrinology. The rise in telemedicine during COVID-19 has made conduct of physical examinations more challenging, especially for pubertal assessment. Previous studies on validity of pubertal self-staging versus clinical examination have yielded mixed results. This study aimed to determine validity and reliability of self-staging of puberty, with potential application during telemedicine visits. The present study is the first to assess pediatric self-rated pubertal staging during the pandemic.

Methods: The study included patients aged 7-22 years referred to Pediatric Endocrinology for specialty care, including pubertal staging. At clinic check-in, patients received a packet with study description, an option to "opt in" or "opt out", sex-specific self-staging instructions, and Tanner (T) stage illustrations. Males received materials for pubic hair (PH) stages T1-T5; females received materials for PH and breast (BR) stages T1-T5. Patients who opted in had 10 minutes to select the image(s) that best matched their bodies, which they sealed in an envelope. This was followed by a clinic visit, where a board-certified pediatric endocrinologist conducted a physical examination, including breast staging (females), testicular size measurement (males), and pubic hair staging (both sexes). Pubertal stage Kappa statistics with 95% CI were calculated for each body part by sex, with Kappa $\geq$ 0.60 indicating significant agreement between self-assessment and physician assessment (0.40-0.60 moderate; 0.20-0.40 fair).

Results: Of 516 distributed packets, 243 self-assessments (125 females) were returned, with 81% (94 females/102 males) being complete (including pediatric endocrinologist staging). Mean age of participants was 12.8 years. Mean BMI was 22.2 kg/m^2 (males) and 23.7 kg/m^2 (females). Hypothyroidism was the most common endocrine diagnosis. For females, kappa was highest for BR and PH in T1 (BR 0.65, PH 0.57) and T5 (BR 0.57, PH 0.65). For males, kappa was highest in T1 (0.73) and T2 (0.58). Grouping Tanner stages into prepuberty (T1), early to mid-puberty (T2-T3), and late puberty (T4-T5) showed greater agreement.

Conclusion: Patients can reliably distinguish between "puberty" and "no puberty" using self-staging, though differentiating between later pubertal stages is more challenging. These findings help define the utility and limitations of self-staging during telemedicine visits.

KEYWORDS

puberty, self-staging, telemedicine, COVID, tanner stage

Introduction

Puberty represents a critical period of development characterized by important hormonal, physical, and cognitive changes, with direct influences on overall body physiology and growth. This period significantly impacts overall Pediatric Endocrine care, as the increase of sex steroids (estrogen or testosterone) drives the development of secondary sexual characteristics and influences metabolic and hormonal parameters (1, 2). Moreover, these hormonal shifts extend beyond sexual maturation, affecting multiple systems throughout the body. From metabolism and growth to thyroid regulation, respiratory, and neurological functions, the broad impact of puberty underscores its importance in shaping overall health during this critical developmental stage (3–7) Thus, accurate pubertal staging and monitoring of puberty are essential for early detection of abnormalities and for supporting healthy development in children and adolescents.

Tanner staging is a physical examination method used to assess pubertal development in children, adolescents, and adults, as initially described by Marshall and Tanner (8). This method categorizes the puberty of individuals into stages based on the development of secondary sexual characteristics such as breast development, pubic hair growth, and genital development. The gold standard practice of Tanner staging involves documentation by a trained clinician (9). Classically, Tanner Stages range from Stage 1 (prepubertal) to Stage 5 (adult development), providing an important framework for diagnosing disorders of puberty, including delayed or precocious puberty. Failure to diagnose delayed puberty can lead to reduced adult height, low bone density with a higher risk of osteoporosis, and increased susceptibility to cardiovascular and metabolic disorders later in life (10). Conversely, undiagnosed precocious puberty can lead to short stature, reproductive issues, increased cardiometabolic risk, and psychological challenges associated with early sexual development (11).

In 2020, the global coronavirus disease-19 pandemic, also known as COVID-19, changed the standards of medical practice. While quarantine measures helped reduce transmission of the disease, they also posed significant challenges to physicians' ability to deliver adequate, timely, and effective health care. To address these challenges, telemedicine gained popularity, offering patients healthcare access while minimizing exposure for vulnerable populations (12) The COVID-19 pandemic served as an opportunity to adopt teleconsultation in response to the urgent need for continuity of care and has indefinitely led to reimagining its use in the management of both acute and chronic diseases (13, 14). In pediatric endocrinology, physical examination of the genitourinary system is crucial; however, telemedicine is an inappropriate platform for this part of the physical examination and does not allow for accurate assessment of common endocrine evaluations, such as pubertal staging.

The rise of telemedicine has made it imperative to find a way to conduct clinical assessments of puberty, as neglecting them could result in harm. This raises an important question: how accurate and reliable are patient self-assessments of puberty in this digital age? Previous research has documented the ability of children and adolescents to self-assess their pubertal development using various tools. These tools have included questionnaires with only written descriptions, those supplemented by drawings (with or without additional descriptions), and those that incorporate real-life photography (with or without written descriptions) (15–19). A number of studies have explored the accuracy of self-staging puberty compared to clinical examinations, noting that results can vary based on the patient's age, self-assessment skills, race, and cultural background (20, 21). Overall, the literature presents inconclusive views on the validity of these self-assessments.

There are clear challenges with using telemedicine for physical evaluations, particularly in pediatric endocrinology where monitoring growth and physical development is important. The aim of this study was to determine validity and reliability of patients' self-staging pubertal assessments for potential use at home during telemedicine visits. To our knowledge, this study is the first to evaluate self-rated pubertal staging by children and adolescents during the COVID-19 pandemic.

Materials and methods

This was a cross-sectional study conducted over 13 months (October 2021-November 2022) at Cincinnati Children's Hospital Medical Center (CCHMC), involving pediatric patients ages 7-22

years who were referred to our tertiary pediatric endocrinology center The study received approval from the Institutional Review Board, and verbal parental consent along with participant assent was obtained. The inclusion criteria focused on those patients for whom pubertal assessment was deemed necessary by their Pediatric Endocrinologist. Exclusion criteria included a known diagnosis of cognitive developmental delay or gender dysphoria.

Upon clinic check-in, eligible patients and/or their guardians received a study packet from an Endocrine nurse containing several key documents: a detailed explanation of the study's procedures, objectives, and rationale; a section allowing patients to voluntarily "opt in" or "opt out" of the study; and written, sex-specific instructions on performing self-pubertal staging. These instructions were accompanied by gender-appropriate illustration sheets depicting the Tanner stages (T1-T5) for both pubic hair (PH) and, for girls, breast development (BR) (17) [Supplementary Materials 1–4]. Of note, male participants were not asked to self-stage testicular size. Those who opted to participate marked "opt in" on the informational sheet and informed the nurse, who then escorted them to the clinic room with their packet.

Patients completed the self-assessment in the absence of the healthcare provider. They had up to 10 minutes to review the illustrations and self-assess their pubertal stage by selecting the Tanner Stage illustration that they felt most closely resembled their current stage of pubertal maturation. Patients then marked their selections on the provided papers, which were then sealed in an envelope with the patient's medical record number (MRN) for later correlation with clinician results. The sealed envelope was handed to the nurse, who placed it in a secure location for delivery to the study team.

Following the self-assessment phase, participants underwent a standard clinic visit, with a complete physical examination by a board-certified pediatric endocrinologist including breast staging for girls, and pubic hair assessment for both sexes. The physical examination took place on the same day as the clinic visit.

For data retrieval, the patient's clinic visit, including the physical examination by the endocrinologist, was accessed from the electronic medical record system using the patient MRN on the envelope. Study data were collected and managed using REDCap electronic data capture tools hosted at CCHMC (22, 23). The recorded data included date of clinic appointment, date of birth, calculated age, sex, race or ethnicity, weight (in kg) with percentile and z-score for age, height (in cm) with percentile and z-score for age, body mass index, any comorbidities or relevant past medical history, medications, self-assessed breast score (for females), self-assessed pubic hair score (for both females and males), physician-assessed Tanner stage for breast development (for females), and physician-assessed Tanner stage for pubic hair (for both females and males).

All demographic information and any patient identifiers were kept in a secure location and only accessible to those involved with the study. After all data were entered in REDCap, the envelope was discarded. To ensure privacy and confidentiality, patients were identified only by numbers in REDCap.

Statistical analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC). Prior study results were used to estimate the required sample size for significance (17). We would require at least 80 participants of each sex to achieve significance, preferably with 16 participants within each Tanner Stage.

Baseline characteristics of the study population were described using means and standard deviations for continuous variables, and percentages for categorical variables. These descriptive statistics were reported for the overall population as well as separately for each sex.

Agreement between self-staging and endocrinologist staging was assessed using the weighted kappa statistic, with 95% confidence intervals (CI) calculated for each sex and body site and stratified by Tanner Stage. Additionally, a pubertal stage kappa statistic was computed to evaluate agreement between self-staging and endocrinologist staging for breast and pubic hair development, with separate 95% CIs for each sex. Kappa values were interpreted as follows: ≥0.6 indicated substantial agreement, 0.4-0.6 indicated moderate agreement, and 0.2-0.4 indicated fair agreement (24). Additionally, we conducted a separate analysis using a simplified grouping of pubertal stages, categorizing them as pre-puberty (Tanner Stage 1), early to mid-puberty (Tanner Stages 2 and 3), and late puberty (Tanner Stages 4 and 5). This approach aimed to assess whether the accuracy of self-staging could be enhanced by utilizing broader pubertal stage categories.

Results

Participant demographics and clinical characteristics

From the 516 packets distributed, 243 self-assessments were received, representing a response rate of 47.1%. Of them, 196 (80.6%) were deemed complete, inclusive of both self-reported pubertal assessments and endocrinologist Tanner staging, with gender representation remaining relatively balanced (48.0% females) (Table 1). The mean (± SD) age of participants at the time of clinical examination was 12.5 ± 2.8 years, age 11.8 ± 3.3 years for females and 13.1 ± 2.1 years for males (Table 2). Notably, analysis of body mass index (BMI) revealed discernible gender differences, with males displaying a mean (± SD) BMI of 21.9 ± 6.4 kg/m^2, while females exhibited a slightly higher mean BMI of 22.7 ± 7.4 kg/m^2. Of all respondents who had a complete endocrinologist staging, 46.4% had high BMI (greater than or equal to the 85th percentile), with 45.1% (n=46) of males and 47.9% (n=45) of females falling into this category.

The majority of participants in this study were White (79%), followed by Black or African American (14%) and Asian (4%); the remaining 3% were Hispanic, Native Hawaiian or Other Pacific Islander, American Indian or Alaska Native, or identified as Other. Participants were being seen in the endocrine clinic for their primary endocrine diagnoses (many had more than one endocrine diagnosis). Thyroid disorders (34.3%) and growth disorders (31.6%) were the most prevalent diagnosis, followed by pubertal disorders (28.3%) and obesity-related conditions (16.9%). Other less common diagnoses included adrenal disorders, bone and calcium disorders, hypopituitarism and others. Notably, some

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TABLE 1 Self-Assessments of breast and pubic hair development (tanner stages 1–5) versus endocrinologist tanner staging.

Endocrinologist Tanner Staging	Self-Assessment N (%)					Total *N (%)*
	1	2	3	4	5	
Breast stage						
1	11 (11.7)	6 (6.4)	0 (0)	0 (0)	0 (0)	17 (18.1)
2	0 (0)	11 (11.7)	1 (1.1)	2 (2.1)	0 (0)	14 (14.9)
3	3 (3.2)	12 (12.8)	14 (14.9)	2 (2.1)	0 (0)	31 (32.9)
4	0 (0)	1 (1.1)	3 (3.2)	3 (3.2)	1 (1.1)	8 (8.5)
5	0 (0)	0 (0)	2 (2.1)	10 (10.6)	12 (12.8)	24 (25.5)
Total	14 (14.9)	30 (31.9)	20 (21.3)	17 (18.1)	13 (13.8)	94 (100)
Females						
Pubic hair stage						
1	11 (11.7)	5 (5.3)	2 (2.1)	1 (1.1)	0 (0)	19 (20.2)
2	4 (4.3)	11 (11.7)	4 (4.3)	0 (0)	0 (0)	19 (20.2)
3	0 (0)	7 (7.5)	6 (6.4)	7 (7.5)	0 (0)	20 (21.3)
	0 (0)	1 (1.1)	2 (2.1)	5 (5.3)	1 (1.1)	9 (9.6)
5	0 (0)	1 (1.1)	0 (0)	10 (10.6)	16 (17.0)	27 (28.7)
Total	15 (16.0)	25 (26.6)	14 (14.9)	23 (24.5)	17 (18.1)	94 (100)
Males						
Pubic hair stage	1	2	3	4	5	
1	27 (26.4)	11 (10.9)	0 (0)	0 (0)	0 (0)	38 (37.2)
2	0 (0)	17 (16.8)	2 (2.0)	1 (1.0)	0 (0)	20 (19.8)
3	0 (0)	2 (2.0)	8 (7.9)	3 (3.0)	4 (4.0)	17 (16.8)
4	0 (0)	0 (0)	1 (1.0)	10 (9.9)	4 (4.0)	15 (14.9)
5	0 (0)	0 (0)	0 (0)	5 (5.0)	7 (6.9)	12 (11.9)
Total	27 (26.4)	30 (29.7)	11 (10.9)	19 (18.8)	15 (14.9)	102 (100)

participants also had additional co-morbidities, including mood disorders (16.3%), neurologic disorders (15.9%), and ADHD/attention disorders (13.4%). The top three medications prescribed were for thyroid disorders (29.5%), mood/behavior/development disorders (20.2%), and growth disorders (11.8%).

Agreement in pubertal staging between physician and patient

The distribution of agreement between endocrinologists' assessment and self-assessment are shown in Table 1. Among females, the highest level of agreement with endocrinologists was observed at Tanner stage 1 (T1) for breast development (kappa = 0.65, 95% CI 0.44-0.86) (Figure 1A) and pubic hair development (kappa = 0.57, 95% CI 0.35-0.79) (Figure 1B), as well as at Tanner stage 5 (T5) for both breast (kappa = 0.57, 95% CI 0.37-0.77) and pubic hair (kappa = 0.65, 95% CI 0.47-0.83). Conversely, the lowest level of agreement with endocrinologists was observed at Tanner stage 2 (T2) for breast (kappa = 0.37, 95% CI 0.18-0.57) and Tanner stage 4 (T4) for breast (kappa = 0.14, 95% CI −0.09-0.37). Interestingly, higher BMI percentile (>85th percentile) was associated with higher kappa scores for breast (kappa = 0.74, 95% CI 0.63-0.86) and pubic hair (kappa = 0.71, 95% CI 0.60-0.84) compared to lower BMI percentile (< 85th percentile) for breast (kappa = 0.52, 95% CI 0.37-0.66) and pubic hair (kappa = 0.58, 95% CI 0.44-0.72).

Among males, the highest level of agreement with endocrinologists was observed at Tanner stage 1 (kappa = 0.73, 95% CI 0.59-0.87) (Figure 1C) and Tanner stage 2 (kappa = 0.58, 95% CI 0.59-0.87) for pubic hair. Conversely, the lowest level of agreement was observed at Tanner stage 5 pubic hair (kappa = 0.45, 95% CI 0.18-0.70). The same kappa agreement was observed at both Tanner 3 and 4 pubic hair (kappa = 0.51). In contrast to the pattern observed in females, higher BMI percentile was associated with lower kappa scores (kappa = 0.69, 95% CI 0.56-0.80) for pubic hair compared to lower BMI percentile (kappa = 0.82, 95% CI 0.73-0.91).

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TABLE 2 "Opt in" study population characteristics.

	Males (n=102)	Females (n=94)
	Mean ± Standard Deviation	
Age, years	13.1 ± 2.1	11.8 ± 3.3
Height, cm	151.1 ± 14.9	146.6 ± 14.5
Weight, kg	51.6 ± 22.1	50.9 ± 24.4
BMI, kg/m2	21.9 ± 6.4	22.7 ± 7.4
BMI Percentile	60.1 ± 37.1	70.0 ± 30.7
BMI >85th percentile (%)	45.1%	47.9%
Most Prevalent Primary Endocrine Diagnoses Among All Sexes (%)*		
Thyroid Disorders	34.3	
Growth Disorders	31.6	
Pubertal Disorders	28.3	
Obesity-related Disorders	16.9	

*Many patients had more than 1 diagnosis.

When examining agreement of physician Tanner Staging and self-staging of puberty categorized as pre-puberty (T1), early to mid-puberty (T2-T3), and late puberty (T4-T5), we observed a notable increase in agreement levels. Specifically, during pre-puberty (T1), breast development in females exhibited a kappa of 0.65 (Figure 2A), while pubic hair development showed a kappa of 0.57 for females (Figure 2B) and 0.73 for males (Figure 2C). In early-mid puberty (T2-T3), the kappa for breast was 0.60, with pubic hair at 0.52 for females and 0.59 for males. In late puberty (T4-T5), agreement for breast was higher (kappa 0.76), with kappa for pubic hair reaching 0.74 for females and 0.79 for males.

Discussion

This study compared self-staging of puberty by children and adolescent patients with assessments made by a pediatric endocrinologist, and evaluated accuracy of self-assessments in pubertal development. The primary goal was to determine the validity and reliability of self-staging of puberty for potential use during telemedicine visits.

Our study found that among females, the highest levels of agreement with endocrinologists were observed at the initial (Tanner stage 1) and final (Tanner stage 5) stages of breast and pubic hair development. This indicates that at the very beginning and end of puberty, females are more capable of accurately self-assessing their development. In contrast, the lowest agreement was noted during Tanner stages 2 and 4 for breast development, suggesting increased difficulty in self-assessment during the intermediate stages of puberty. For males, the highest agreement with endocrinologists was seen at Tanner stages 1 and 2 for pubic hair development, similar to females, indicating that self-assessment appears to be most reliable at the onset of pubertal maturation. Conversely, for males the lowest agreement was observed at Tanner stage 5, with intermediate stages (Tanner 3 and 4) showing moderate agreement levels.

When examining agreement levels by broader pubertal categories, we found an overall improvement in agreement when grouping Tanner stages into pre-puberty (T1), early to mid-puberty (T2-T3), and late puberty (T4-T5). Specifically, pre-puberty stages showed improved agreement for both breast and pubic hair development across genders. Agreement levels remained moderate during early to mid-puberty, and significantly increased during late puberty. These findings indicate that a broader categorization may simplify the self-assessment process and improve its accuracy. Additionally, the data suggest a consistent pattern of increasing agreement during the later stages of puberty, highlighting a trend towards greater concordance as pubertal maturation progresses.

Interestingly, the current study found that a higher BMI percentile was associated with higher agreement scores for both breast and pubic hair development among females. This was surprising to us, as prior research suggested that elevated BMI and higher levels of body fat can hinder the ability to differentiate between lipomastia and true breast tissue, which may result in an exaggerated assessment of breast development (25). This is also in contrast with previous literature that found that BMI did not appear to significantly influence self-assessment (16). Notably, the earlier study featured a higher proportion of female participants with lower weights and BMIs, whereas nearly 50% of the females in our study had BMIs above the 85th percentile. This difference in BMI distribution may explain the discrepancy between our findings and those of the earlier research. Nonetheless, it appears that accuracy was not confounded by being overweight, indicating that self-assessment reliability can still be achieved across different BMI percentiles. In contrast, males with higher BMI percentiles exhibited lower agreement scores for pubic hair development. We did not assess gonadarche in males and thus we do not know if self-assessment of gonadarche is confounded by being overweight in male patients. Further research is justified.

There are some limitations affecting this study. Not all individuals who self-evaluated their Tanner Stage received a "gold standard" physical assessment by a pediatric endocrinologist. Specifically, for 47 patients, no Tanner stage examination was documented in the electronic medical record. It remains unclear whether the exam was not conducted, not documented, or declined by the patient. Additionally, the endocrinologist may have determined that the pubertal exam was not clinically relevant for that specific visit. As a result, we may have missed possible instances of concordance or discordance between self-assessments and expert appraisals. There was also an unequal distribution and representation of Tanner stages among participants, which may have impacted the generalizability of our findings. Finally, male participants were not asked to self-assess testicular size, which is an integral component of accurately evaluating pubertal status. While we considered including testicular measurements, we determined that it could impose additional burden on both participants and the study process. Collecting such data might have introduced complexity and extended the duration of examinations, potentially affecting participant compliance and the overall

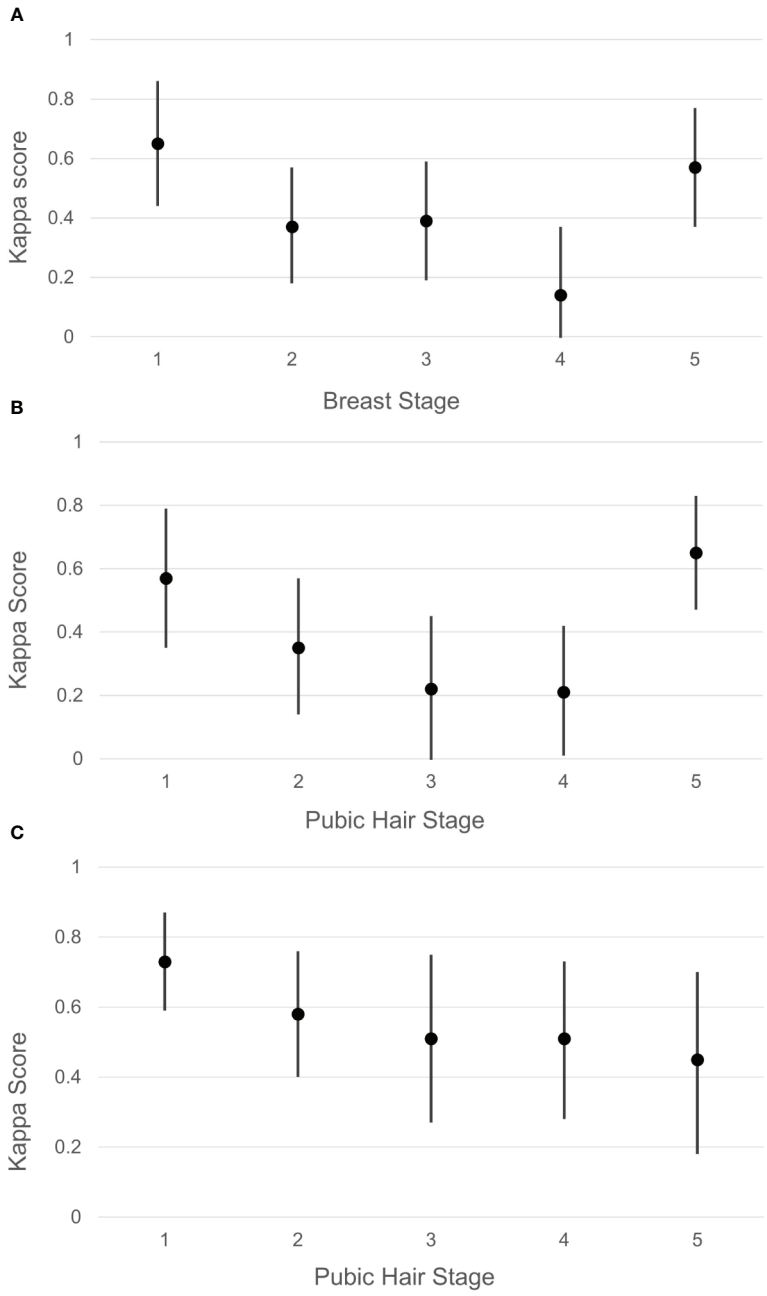

FIGURE 1
Agreement (represented by Kappa score and 95% confidence interval) between pubertal self-staging and pediatric endocrinologist assessment by Tanner stage for patients seen in endocrinology clinic. **(A)** Female breast stage. **(B)** Female pubic hair. **(C)** Male pubic hair.

feasibility of the study. Therefore, we made the decision to focus on other markers of pubertal maturation that could be more readily and consistently measured across participants using visual, not tactile, cues. However, this omission may have resulted in incomplete or inaccurate assessments of male pubertal development.

Despite these limitations, our study has several strengths. A key strength was that the gold standard physical examination was conducted by a pediatric endocrinologist who was blinded to the patient's self-assessment. Both the physician examination and the patient's self-staging were performed on the same day, ensuring consistency. Additionally, a large cohort of patients submitted self-assessments, providing robust data. Lastly, the study population was diverse, representing a range of racial backgrounds, primary endocrine diagnoses, and anthropometric measurements.

Conclusion

We compared self-staging of puberty by children and adolescent patients with the Tanner stage physical examination made by a pediatric endocrinologist, evaluating the accuracy of self-

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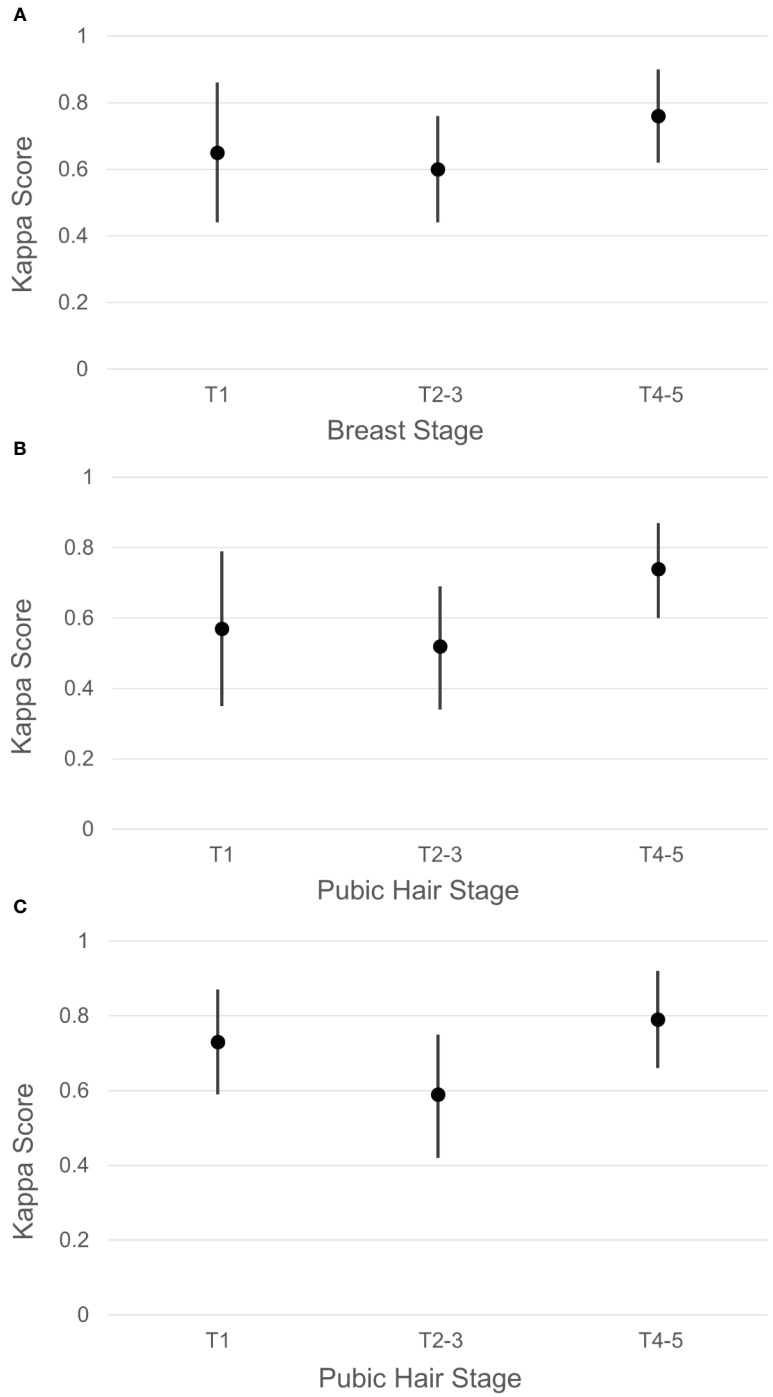

FIGURE 2
Agreement (represented by Kappa score and 95% confidence interval) between pubertal self-staging and pediatric endocrinologist assessment by grouped Tanner stages (pre-puberty T1, early to mid-puberty T2 to T3, or late puberty T4 to T5) for patients seen in endocrinology clinic. **(A)** Female breast stage. **(B)** Female pubic hair. **(C)** Male pubic hair.

assessments in pubertal maturation. The findings indicate that, in general, children and adolescents can accurately distinguish between "puberty" and "no puberty" using self-staging, although differentiating between individual pubertal stages is less reliable. A greater level of agreement was observed when female and male Tanner stages were grouped into pre-puberty, early to mid-puberty, and late puberty categories. Thus, pubertal self-staging can potentially serve as a valuable and efficient clinical tool, offering a viable alternative to in-person physical examinations, particularly when patients are unable to attend clinic visits. Consequently, this study contributes to understanding the utility and limitations of self-staging during telemedicine visits, highlighting its potential role in clinical practice particularly as telehealth continues to be an important part of healthcare post-pandemic (26).

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 humans were approved by the Cincinnati Children's Hospital Institutional Review Board (IRB). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because the study was considered to present minimal risk to the patient. We secured verbal assent and consent from both the patients and their parents or caregivers. Upon clinic check-in, the parent and child received a concise, half-page summary outlining the study's procedures and rationale. If they chose to participate, they marked "opt in" on the sheet and informed the nurse, who then proceeded with guiding them to the clinic room for continued participation in the study.

Author contributions

CE: Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. JM: Writing – review & editing, Resources, Investigation, Data curation. KB: Writing – review & editing, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. SR: Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization. NY: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

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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

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1487329/full#supplementary-material

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Endocrine disorders in Rett syndrome: a systematic review of the literature

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Background: Rett syndrome (RTT) is an X-linked progressive neurodevelopmental disorder that involves mainly girls and is the second most frequent cause of genetic intellectual disability. RTT leads to neurological regression between 6 and 18 months of life and could be associated with a variable neurological impairment. However, RTT affects not only neurological function but also wide aspects of non-neurological organs. Recent data showed that the endocrine system is often involved in RTT patients, including disorders of growth, bone health, thyroid, puberty onset, and weight abnormalities However, systematic data on endocrinopathies in RTT are scarce and limited.

Objective: This review aims to analyze the prevalence and type of endocrine comorbidities in RTT population, to allow a precocious diagnosis and appropriate endocrinological management.

Methods: Systematic research was carried out from January 2000 to March 2024 through MEDLINE via PubMed, Scopus, and the Cochrane Library.

Results: After the selection phase, a total of 22 studies (1090 screened) met the inclusion criteria and were reported in the present review. Five studies were observational-retrospective, four were cross-sectional and case report or series, three were survey, prospective, and case-control, and finally one study for descriptive-transversal and longitudinal population-based study. The sample population consisted of multiethnic groups or single ethnic groups. The main endocrinopathies reported were malnutrition, bone alterations, and alterations of puberty onset.

Conclusions: Our analysis shows that endocrinopathies are not rare in RTT patients. Therefore, in the context of a multidisciplinary approach, accurate screening and monitoring for endocrinopathies should be recommended in all RTT patients, to improve clinical practice, healthcare management, and, finally, patients' quality of life.

KEYWORDS

Rett syndrome, endocrinopathy, MECP2 deletion, CDKL5 deletion, epilepsy

1 Introduction

Rett syndrome (RTT; OMIM ID 312750) is a severe neurodevelopmental disorder that has been identified almost exclusively in females, mainly after 6 months of age (1). It affects about 1 in 15,000 newborns (2). It is the second genetic cause of intellectual disability in girls after Down syndrome (3). In 90–95% of the cases, mutations in Methyl-CpG binding protein 2 gene are responsible for most typical RTT and a smaller proportion of atypical RTT. On the other hand, patients with Rett phenotype together with early-onset epilepsy caused by mutations in the cyclin-dependent kinase-like 5 gene (*CDKL5*) (4). Another gene known as *FOXG1* has been associated with atypical RTT or a RTT-like phenotype, and may manifest with preserved function and specific clinical features (5). In 1999 the first mutations in the methyl-CpG binding protein-2 (*MeCP2*) gene were described. The *MeCP2*gene codes for the methyl-CpG binding protein-2 (*MeCP2*) which is involved in the long-term silencing of genes and is expressed in all tissues (6). Mutations in the *MeCP2*gene, primarily causing a loss of function, are mainly responsible for RTT, a disorder affecting the X chromosome (7). Since roughly 95% of the mutations occur (*de novo*), prenatal testing and/or genetic counseling for Rett syndrome is often not helpful. *MeCP2* plays a pivotal role in brain functioning and neuronal development, both at the beginning of neuronal differentiation and thereafter (8).

RTT patients begin life apparently 'healthy'. However, from 6 to 18 months of age, they undergo regression of early milestones, with deterioration of motor skills, eye contact, speech, and motor control, deceleration of head growth, and development of distinctive repetitive, purposeless hand movements (9). A spectrum of neurological issues, including anxiety, breathing problems (respiratory dysrhythmias), and seizures, usually develop over time (10). The clinical phenotype of RTT is highly variable and can be classified into two main categories: typical (classic) RTT and atypical (variant) RTT. Diagnostic criteria for typical RTT require a period of regression, followed by recovery or stabilization, and fulfillment of all the main criteria (loss of purposeful hand skills, loss of spoken language, gait abnormalities, and stereotypic hand movements) (3). Further manifestations can include autistic features, intermittent breathing abnormalities, autonomic nervous system dysfunction, cardiac abnormalities, and sleep disturbances. In addition to typical or classical RTT, some individuals may present with many, but not all the clinical features of RTT, thus there are 'variant' or 'atypical' RTT (11). These include three main variants: preserved speech, early onset seizure, and congenital variants (12). Trofinetide is at the moment the only disease-modifying therapy for RTT approved by FDA since 2023, and it is a potential effective and safe therapeutic opportunity (13). Different pharmacologic drugs, including glatiramer acetate and dextromethorphan have been investigated in small clinical trials but with modest benefits (14). Gene therapy, which is nowadays in the drug development phase, may promise new cure opportunities (15).

Initially, RTT was considered a purely nervous system pathology, but in recent years it has emerged as a complex and heterogeneous multisystemic disease, with a variety of clinical appearances (16). Although neurological conditions are predominant, the disease affects not only the central nervous system but also a wide array of non-neurological organs. Recent studies showed that multisystemic comorbidities, like gastrointestinal, orthopedic, endocrine, or cardiac issues, may be more or less prevalent in RTT patients (17). Concerning endocrine disorders in RTT, data are few and contrasting. Even if endocrinopathies are less common among comorbidities, they seem to be significantly more frequent than in the general population. Endocrine disorders have a considerable impact mainly on growth, weight, menstrual cycles, and bones. Some authors reported low bone mineral content as the most common endocrine disorder in RTT (18), followed by alterations in the timing of pubertal onset and menarche (19). Furthermore, thyroid function is a matter of great concern in these patients, considering the effect of thyroid hormones (TH) on proper mammalian brain development (20).

Overall, data on endocrinopathies in RTT patients is still scarce and univocal. This systematic review aims to describe the prevalence and type of the main endocrine comorbidities, focusing especially but not exclusively on pediatric age, providing a proposal for endocrinological management of RTT patients.

2 Methods

We performed the review following the PRISMA 2020 guidelines (21). Systematic research was performed, covering the last 24 years (from January 2000 to March 2024), according to the PRISMA statement, through MEDLINE via PubMed, the Cochrane Library, and Scopus databases, to find studies reporting endocrinopathies in patients with genetic diagnosis of Rett syndrome. The research was based on the combination of "Rett syndrome" with all the following keywords: "thyroid", "growth", "short stature", "obesity", "malnutrition", "puberty", "menstruation", "menstrual irregularities", "hyperprolactinemia" and "bone" to include a variety of results. No specific registers of RTT population were used for the systematic research. Literature before 2000 was not included because the main causative genetic mutation in RTT was identified in 1999 (7, 22).

The settled inclusion criteria were: articles written in English, belonging to the categories of a review, clinical study, clinical trial, clinical trial protocol, multicenter study, randomized controlled trial, and observational study, which report endocrinopathies in genetically confirmed RTT in pediatric or mixed population (both adolescents and adults patients). Due to the rarity of studies in this field, case reports and small cohorts were also included. The exclusion criteria were letters to editors, articles belonging to only the adult population, not full-text articles, absence of genetic diagnosis of RTT and experimental studies (for example murine studies). From our initial research, all the studies reporting the presence of one or more endocrinopathies in RTT patients were subsequently reviewed individually, hence, we focused on: the type of endocrinopathy, incidence and/or prevalence, specific gene

mutation, presence or absence of epilepsy, and eventually anticonvulsant drugs.

Titles and abstracts of all retrieved articles were screened by four authors (R.C., G.L., C.L., T.A.) to identify articles for full-text review. All authors assessed the eligibility of all full-text articles.

2.1 Data items

The following information was extracted from the included studies: bibliographic data (first author, publication year, country), study characteristics (study design), participant characteristics (sample size, gender, age at onset, Rett mutation, epilepsy), outcome (prevalence of endocrinopathies). Equations

3 Results

We identified 1750 records. After duplicates and not in English full-text removal, we screened 1090 records, from which were viewed 1090 full-text studies, and finally included 22 articles (19, 23–43) after title, abstract, and PICO evaluation (Figure 1). As above mentioned, we did not carry out meta-analyses since the studies included were not homogeneous in terms of the type of study design, number of cohorts, age, and race difference among populations. Five studies were observational-retrospective, four were cross-sectional, three were case reports or series, three were survey, three were prospective, two comprehended case-control, and finally one study for descriptive-transversal and longitudinal population-based study. The sample population consisted of multiethnic groups or single ethnic groups. Nine studies (40.90%) were from the USA, seven (31.82%) from Europe, three (13.64%) from Australia and the remaining three studies (13.64%) were from Taiwan, Japan, and Brazil. Thirteen (59.10%) studies were published after 2012. Only two studies included male patients together with a female population. Seven studies included exclusively RTT patients < 18 years old while the other articles involved both adults and adolescents. Interestingly only one study included FOX1 gene mutation together with *MeCP2* and *CDKL5* mutations, and two studies reported patients with *CDKL5* mutation and *MeCP2*. The main endocrinopathies described in the selected studies were malnutrition and bone alterations (8/24) followed by puberty onset disorders and obesity (7/24). Short stature was identified in 6/24 studies, menstrual irregularities in 5/24, thyroid disorders were presented in 2/24 studies, and hyperprolactinemia in only 1/24 study. Finally, seizure disorders were described as RTT comorbidities in fourteen studies (63.60%). The main characteristics of the included studies are reported in Table 1.

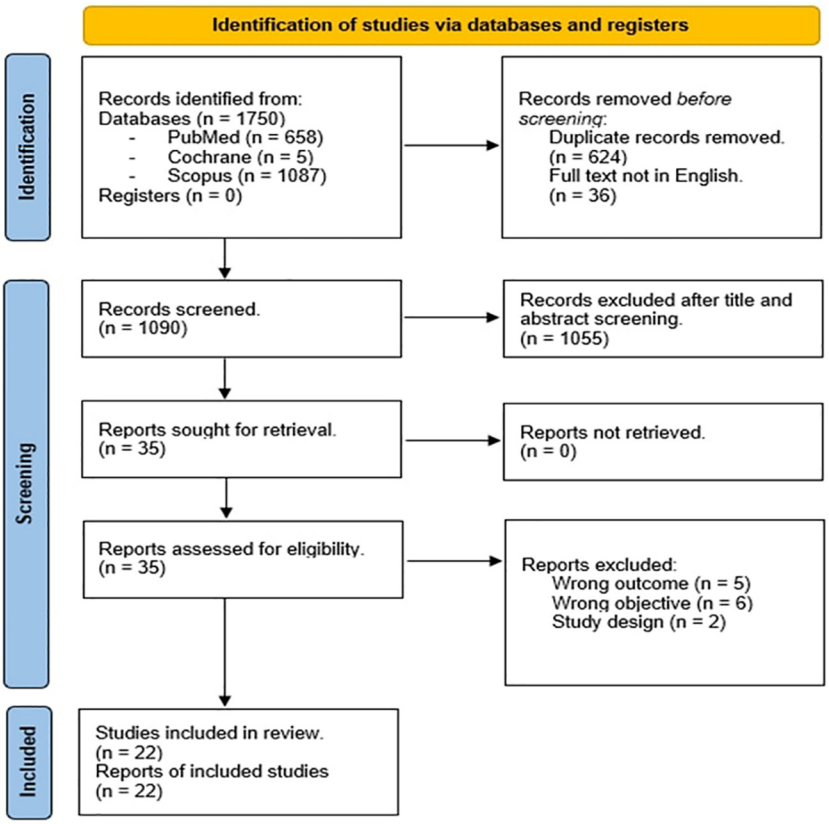

FIGURE 1
PRISMA 2020 flow diagram of the study

10.3389/fendo.2024.1477227

TABLE 1 The Table summarizes the main data of the studies reporting endocrine disorders in Rett syndrome patients.

PAPER, YEAR, COUNTRY	DESIGN	N. PATIENTS	AGE OF EVALUATION	SEX/ RACE	GENETIC	ENDOCRINOPATHIES	EPILEPSY
Pepe et al., 2024 (23) **Italy**	Retrospective observational study	51	9.65 ±5.9 years	47 *female*, 4 *male* *Caucasian*	*MeCP2* (74.5%), *CDKL5* (21.6%), *FOXG1* (3.9%)	*Short stature* (47.1%), *Menstrual irregularities* (46.2%), *Malnutrition* (25.5%), *Obesity* (19.6%), *Bone alterations* (19.6%), *Precocious puberty* (15.4%), *Hyperprolactinemia* (13.7%), *Thyroid alterations* (9.8%).	*Epilepsy disorders* (72.5%)
Stagi et al., 2014 (24) **Italy**	Case-control Control: 146 age-matched healthy Caucasian girls	45	8.6 ± 5.3 years	45 *female*, 0 *male* *Caucasian*	*MeCP2* (97%), *CDKL5* (13%)	*Thyroid alterations*: - 17.7% showed FT4 levels higher than the upper references limit. - 26.7% showed FT3 levels higher than the upper references limit - 10.2% showed TSH high levels	*Epilepsy disorders* (29%)
Wong et al., 2021 (25) **Taiwan**	Cross-sectional	44	17.03 ± 10.52 years 54.55% <18 years	44 *female*, 0 *male* *Taiwanese*	*MeCP2* (100%)	*Short stature* 41.6% *Malnutrition* 37.5% *Obesity* 4.1%	*N/A*
Huppke et al., 2001 (26) **Germany**	Prospective	38	Median 7.1 years (range 2.1 to 17 years)	38 *female*, 0 *male* *Caucasian (from Germany)*	*N/A*	*Short stature* 91.1%	*N/A*
Tarquinio et al., 2012 (27) **USA**	Observational study From the multicenter RTT Natural History Study (RNHS)	816	345 <10 yo 252 > 10 yo	816 *female*, 0 *male* *Caucasian* 88% *African American* 6% *Asian* 5% *American Indian* 1%	*MeCP2* (92%)	Severe somatic growth deficit with microcephaly (29.2%) *Precocious puberty* 12% *Late-onset puberty* 3%	*N/A*
Reilly et al., 2001 (28) **Australia**	Retrospective Observational study	59	2.1-44.9 years old (Mean: 12.6 years SD 11.04 years).	59 *female*, 0 *male* *Caucasian*	*N/A*	*Malnutrition* 26%	*Epilepsy disorders* (62.7%)
Schwartzman et al., 2008 (29) **Brazil**	*Descriptive and transversal study*	27	Between the ages of 2.6 and 21.8 years	27 *female*, 0 *male* *Brasilian*	*N/A*	*Short stature* 48.1% *Malnutrition* 37% *Obesity* 7.4%	*Epilepsy disorders* (69.2%)
Czerwonogrodzka-Senczyna et al., 2023 (30) **Poland**	*Case-control Control: 22 healthy female*	49	8.7 ± 4.9 years	49 *female*, 0 *male* *Polish*	*Common MeCP2 mutations 51%*	*Malnutrition* 38.78% *Obesity* 10.2%	*N/A*
Motil et al., 2012 (31) **USA**	*Survey* From the North American RTT database	983	0–5 y: 12%; 6–10 y: 22%; 11–14 y: 12%; 15–19 y: 17%; 20–29 y: 24%; 30+ y: 13%	983 *female*, 0 *male* N/A	*MeCP2 mutations 87%*	*Short stature* 45% *Malnutrition* 38% *Bone diseases* 37% *Obesity* 9%	*Epilepsy disorders* (81%)
Knight et al., 2012 (32) **Australia**	*Longitudinal population-based data* From the Australian population-based Rett Syndrome Database.	213	Born from 1976	213 *female*, 0 *male* N/A	*MeCP2 mutations 100%*	*Malnutrition* 21.5% *Delayed menarche* 9% *Precociuos puberty 6%* *Obesity* 4.2%	*N/A*

(Continued)

TABLE 1 Continued

PAPER, YEAR, COUNTRY	DESIGN	N. PATIENTS	AGE OF EVALUATION	SEX/ RACE	GENETIC	ENDOCRINOPATHIES	EPILEPSY
Baş et al.,2013 (33) **Turkey**	*Case report*	1	6 years	1 female Turkish	*MECP2: C455G P152R,* missense mutation	*Precocious puberty*	*Epilepsy disorders*
Killian et al., 2013 (19) **USA**	*Retrospective observational* Through the multicenter RTT Natural History study (RNHS)	802	Born from 1943 to 2010	802 *female*, 0 *male* *American Indian* 0.7% *Asian* 4.4% *Native Hawaiian* 0.3% *Black* 4.2% *White* 86.9% *Mixed black and white* 1.6% *Missing demographic data* 1.9%	*MeCP2 mutations 100%*	*Delayed menarche* 19% *Premature menarche* 13% *Precociuos puberty 10%*	*N/A*
Bernstein et al., 2019 (34) **Germany**	*Case series*	2	11 and 34 years	2 *female*, 0 *male* *Caucasian*	*A* novel *MeCP2 a* variant: *c.1162_1172del; p.Pro388* in both patients	*Precocious puberty*	*Epilepsy disorders* (100%)
Yang et al., 2021 (35) **China**	*Case report and literature review*	1	8 years	1 *female*, 0 *male* *Chinese*	*De novo MeCP2 variant c.1157_1197del*	*Precocious puberty*	*Epilepsy disorders*
Hamilton et al., 2012 (36) **USA**	*Anonymous web-based survey.*	21	10-25 years (average 17.1)	21 *female*, 0 *male* *Caucasian 86%*	*N/A*	*Premenstrual Syndrome* 71% *Dysmenorrhea 76%*	*N/A*
Humphrey et al., 2020 (37) **USA**	*Retrospective cross-sectional chart review and prospective survey.*	77	12-55 years	77 *female*, 0 *male* *Black or African American* 9% *Caucasian* 88% *Other, not specified* 3%	*MeCP2 64.9%* *Unknown* (not tested/results unavailable/ unknown pathogenicity) 35.1%	*Dysmenorrhea 61%* *Catamenial seizure 22.1%*	*Epilepsy disorders* 61%
Motil et al., 2015 (38) **USA**	*Cross-sectional, prospective*	50	15.5 ± 9.7 y (2-38 y)	50 *female*, 0 *male* *Caucasian* 64 African American 16% Hispanic 14% Asian 6%	*MeCP2 90%*	*Low bone mineral content* 59% *Low bone mineral density* 45% (decreased bone formation rather than increased bone resorption) Osteocalcin concentrations for all age groups were significantly lower, whereas the concentrations of bone alkaline phosphatase J were significantly higher in the RTT cohort than their respective values	*Epilepsy disorders* 66%
Jefferson et al., 2014 (39) **Australia**	*Clinical trial* From the Australian Rett Syndrome Database (ARSD)	97	Mean age 15 yo (4–30.5 years)	97 *female*, 0 *male* N/A	*MeCP2 90%*	*Fractures* 31.7% *Low mineral density:* - *Lombar* 41.3% - *Total* 44.6% - *Femoral* 78%	*Epilepsy disorders* 75.3%

(Continued)

TABLE 1 Continued

PAPER, YEAR, COUNTRY	DESIGN	N. PATIENTS	AGE OF EVALUATION	SEX/ RACE	GENETIC	ENDOCRINOPATHIES	EPILEPSY
Budden et al., 2003 (40) **USA**	*Clinical trial* From the Australian Rett Syndrome Database (ARSD)	5	Mean age 12.05 yo (4–30.5 years)	5 *female*, 0 *male*	*MeCP2 60% Unknown 40%*	*Low bone volume accompanied by low bone formation rates of* 100%	*Epilepsy disorders* 20%
Motil et al., 2011 (41) **USA**	*Retrospective review, observational*	284	11.7 ± 8.4	284 *female*, 0 *male Caucasian* 69% *Hispanic* 19% *African American* 5% *Asian* 5% *Native American* 2%	*MeCP2 99% Unknown 1%*	*Low vitamin D* 20% *Malnutrition* 15% *Obesity* 4%	*Epilepsy disorders* 57%
Shapiro et al., 2010 (42) **USA**	*Cross-sectional observational study*	49 + 1	Female: 1.9–17 y (mean age, 7.6 3.8 y) Male: 6 years	49 *female*, 1 *male* N/A	*MeCP2 100%*	*Low bone mineral density* 48.9% *Fractures* 11%	*Epilepsy disorders* 31%
Caffarelli et al., 2020 (43) **Italy**	*Retrospective* survey	232	age range 4–33years; mean age 13.8 ± 8.3yrs	232 *female* Caucasian	*MeCP2 100%*	*Scoliosis 51.6% Bone fractures 20.9%*	N/A

Selected following the inclusion features of the studies Characteristics of included studies. N/A, not applicable.

4 Discussion

In the last two decades, there has been growing evidence that RTT is not only a neurological disorder but potentially affects several organs and apparatus (44). RTT has a complex and heterogeneous clinical appearance (17). Although neurological conditions are predominant, the disease also affects various non-neurological organs. A possible explanation for this multisystemic involvement may be related to the ubiquitous presence of *MeCP2* in peripheral tissues. Multi-system comorbidities, e.g. gastrointestinal, orthopedic, endocrine, or cardiac issues could be more or less prevalent. Notably, endocrine disorders are more frequent in RTT than in the general population even if systematic data are still limited and not univocal. We selected studies published in the last 24 years, focusing on the prevalence and type of endocrinopathies reported in RTT patients.

4.1 Short stature

Short stature is reported as one of the most common endocrinological findings in RTT population. Indeed, growth failure is among the supportive diagnostic criteria of RTT, usually observed as decreased velocity of head growth, height, and weight for age (3). To the best of our knowledge, this finding was first described in 1992 by Thommessen et al., reporting a stature below the -2 standard deviation scores (SDs) for sex and age in RTT girls (45). Growth velocity is usually normal until 15 months of age when deceleration is first noticed. In 1993 Schultz et al. underlined the pattern of growth of RTT mainly characterized by deceleration of head growth, followed by deceleration of weight and height measurements. Considering that the typical motor and behavioral alterations of RTT often appear after the second year of life, this growth pattern can be the first clinical element of suspicious for this syndrome (46). In consideration of this typical growth pattern in RTT Tarquinio et al. in 2012 proposed specific Growth Charts for height, weight and BMI for patients with Rett syndrome that have been approved and used by International Rett Syndrome Foundation to avoid a common clinical and research setting in RTT (27).

Regarding possible endocrine disruption in RTT involving growth, Huppke et al. showed that growth retardation - which involved 91.1% of their RTT cohort doesn't seem to be caused by Growth Hormone (GH) deficiency. Nevertheless, disturbed hypothalamic control cannot be excluded (26). After about 10 years, even Hara et al. investigated short stature and GH deficiency in RTT. Notably, despite patients with epilepsy and Rett syndrome having a delayed growth spurt, the final height wasn't statistically different from a control group with only epilepsy. Moreover, circulating levels of GH, IGF-1, and ghrelin were not significantly correlated with height in either group (47).

Of course, in RTT nutritional status could negatively affect growth because of the decline in feeding abilities that may occur during ages. Oddy et al. reported that factors such as enteral nutritional support, mobility, breath-holding, and hyperventilation

may strongly affect growth in subjects with RTT. Feeding difficulties could impair adequate nutritional intake, so active nutritional management is needed to ensure RTT patients the best opportunity to reach their growth potential (48).

In this regard, in 2001 Reilly et al. underlined that, even if mechanisms causing growth failure are poorly understood, both nutritional and non-nutritional factors are pivotal (48). In the following years, a lot of studies were published about new mutations in the *MeCP2* gene and their association with phenotype. Tarquinio et al. reported a correlation between the subtype of *MeCP2* mutation with growth pattern, showing that growth failure occurs less frequently in case of late truncation mutations. This finding was probably due to mutations that affected patients with milder phenotypes. In contrast, other types of mutations (pre-C-terminal truncation and R270X), may convey several comorbidities that have an impact on growth in RTT, such as oropharyngeal and gastrointestinal dysfunctions, scoliosis, seizures, and osteopenia (27). Therefore, it seems that mutations are correlated to growth velocity but above all with clinical severity. Indeed, growth failure is more evident in case of mutations that lead to greater clinical severity, such as pre-C-terminal truncation and R270X, rather than that associated with a milder clinic phenotype, such as R306C, R133C, and C-terminal truncation (49). Tarquinio et al. found severe growth failure together with microcephaly in 29.2% of their RTT cohort. Interestingly, Huppke et al. showed that most females with RTT and *MeCP2* mutations had a smaller occipitofrontal circumference, shorter length, and lower weight at birth. Indeed, a role of *MeCP2* protein in intrauterine development could be hypothesized (50). Recently, Wong et al. underlined the correlation between growth deficit, malnutrition, and clinical severity of the disease (age of onset, dystonia, deambulation, hand use, and language impairment), reporting a prevalence of 41.6% for short stature and 37.5% for malnutrition (25). This study was also the first to give importance to ethnicity when comparing RTT individuals' growth patterns to the RTT-specific chart.

Most recently, Pepe et al. carried out a two-center observational study on a pediatric cohort of 51 RTT pediatric patients with different genetic mutations (*MeCP2, CDKL5, FOXG1*). Short stature was reported as the most common endocrine disorder (47.1%), after excluding secondary causes of growth restriction (celiac disease and GH deficiency). Interestingly, all the patients with short stature exhibited *MeCP2* mutations (23).

4.2 Weight disorders (obesity and malnutrition)

Obesity and malnutrition represent the opposite manifestations of an alteration in weight balance in RTT patients. They are both among the most common endocrinopathies reported in RTT, with malnutrition being more prevalent than obesity. *MeCP2* deletion induces dysregulation of lipid metabolism along with significantly increased lipogenic enzyme gene expression and may alter the expression of hypothalamic genes related to feeding regulation. In this direction, an interesting experimental study carried on in 2013 showed that *MeCP2* positively regulates POMC expression in the hypothalamus. The absence of *MeCP2* in POMC neurons leads to increased DNA methylation of the POMC promoter, which, in turn, downregulates POMC expression, leading to obesity in mice with increasing leptin resistance. Therefore, in POMC neurons *MeCP2* seems to be essential in energy homeostasis regulation (51).

Experimental animal models showed that in female heterozygous *MeCP2* -null mice fed with a high-fat diet, a dysregulation of food intake in the hypothalamus and dopamine reward circuitry can be observed, accelerating the development of obesity (52). Another hypothesis correlates genetic *MeCP2* disorders and syndromic manifestations. The first *de novo* mutation of *MeCP2* was described in 2002 in a male patient (53), associated with moderate intellectual disability, hypotonia, obesity, and gynecomastia. Couvert et al. also observed obesity and neurological disabilities in three patients with *MeCP2* mutations (54). Although obesity in these patients could be an occasional finding, it might also suggest a role for *MeCP2* in regulating energy balance. Moreover, some patients with features of Angelman syndrome may also convey mutations in the *MeCP2* gene (55). Nevertheless, the correlation between weight and genotype remains unclear and is mainly investigated in murine studies.

Similarly to short stature, malnutrition is often present in RTT patients in the first years, soon after the onset of neurological regression. Already in 1992, studies on weight and nutrition indicated that underweight was likely to be the consequence of oral-motor dysfunctions and poor self-feeding abilities. These problems, despite preserved appetite, lead to reduced food intake with subsequent growth retardation, with the mean energy intake being 66.9% in RTT according to the US recommendations (56) for age and 107.8% for body weight (45).

Weight deficit in RTT seems to be related to alterations in energy balance (57). In a Polish case-control study of 49 Rett adolescent females, malnutrition was described in 38.78% and obesity in 10.2% of the case group. In comparison to Polish healthy girls, the diet of girls with Rett syndrome was characterized by a significantly lower energy value, and less carbohydrate, protein, fiber, calcium, and iron content. All comorbidities that include feeding disorders requiring crushing, chopping, or blending of food, and depending on the mobility of patients will compromise alimentation and consequently weight status (30). Wong et al. reported that 41.03% of their Taiwanese RTT cohort had inadequate energy intakes, especially in the severe growth deficit group with age $\leq$ 18 years; nevertheless, there was no significant difference regarding the nutrient intake between severe growth deficit and mild or moderate growth deficit. This suggests that other factors in addition to nutrient intake may play a role in the growth pattern of those with severe growth deficit (25). As mentioned above, Wong et al. underlined that nutritional status and dietary intake in RTT may differ among various ethnic groups and countries.

Conversely, several studies reported a normal BMI in RTT patients. This is probably due to concomitant weight and height deficiency that typically affects RTT (27, 31).

Moreover, it should always be kept in mind that *MeCP2* is a ubiquitous protein, expressed even in the gastrointestinal (GI) tract. Alterations in the digestive system could be present and contribute to poor alimentation and underweight. In a cohort of 983 females with

RTT, 92% had GI problems (gastroesophageal reflux, constipation, straining with bowel movements, and passage of hard stools) and a small percentage (4.4%) also have biliary tract disease, which may have a fatal outcome (31). In RTT, disruption of swallowing may occur at any stage causing significant alteration of the eating and drinking phases and resulting in dysphagia (disorders that may occur in the oral, pharyngeal, or oesophageal stage). Still in 2001, when RTT's genetic alterations were almost unknown, Reilly et al. described malnutrition in 26% of 59 Australian RTT girls and concluded that the consequences of dysphagia for both females with Rett syndrome and their caregivers are affecting health, development, and general well-being (28).

While several studies have been published regarding malnutrition in Rett population, little is known about the impact of obesity. According to Motil et al., 9% of female individuals with RTT were diagnosed as overweight or obese (31). Although RTT has been associated with obesity, the underlying mechanism has not been elucidated yet. The prevalence reported is quite similar in different studies, but of course, also ethnicity, social condition, and food availability should be considered as influencing factors. For example, in a transversal Brazilian study by Schwartzman et al., malnutrition affected 37% of RTT females, and obesity 7.4% (29). In a recent pediatric observational Italian study, the prevalence of malnutrition and obesity were respectively 25.5% and 19.6% (23).

Few studies demonstrated weight-for-height excess, as well as overweight among children with autism spectrum disorder, hypothesizing an eating disorder under neurological disorders. Research has shown that children with autism may be more likely to have weight problems, such as being overweight. This could be due to underlying eating disorders associated with neurological conditions (58).

The role of leptin, a peptide hormone mainly produced by white adipose tissue, has been investigated in the regulation of body weight and energy expenditure. Blardi et al. reported higher leptin levels in RTT patients than controls positively correlated with age and BMI (59). Unexpectedly, the increased leptin concentrations were not always associated with obesity. Authors hypothesized that in patients with RTT, leptin levels might be related to factors other than weight balance, such as the regulation of neuroendocrine and immune functions, and infections, frequently present in RTT due to respiratory alterations (55).

The assessment of the nutritional status of the individual with RTT serves as a guide to nutritional intervention such as supplemental formula use, feeding gastrostomy, etc. Regular monitoring of growth parameters, including weight, height, and BMI, is necessary. However, measurements in RTT are sometimes challenging since some patients are unable to stand on their own or have spinal deformities, while the mere comparison of body weight cannot be concluded without reference to body height and height gain.

4.3 Gonadal function

4.3.1 Precocious puberty

Abnormalities in pubertal onset represent a prevalent aspect of endocrine comorbidity in RTT. The most common alteration of puberty in RTT syndrome is precocious puberty (PP). Data about pubertal disorders come mainly from case report series. Interestingly, this finding has been reported more recently than short stature, malnutrition, and bone dysfunctions. Mainly, the relationship between puberty and RTT was evaluated in murine experimental trials. In 2009, Garcia-Rudaz et al. showed that in a mouse model of RTT, the expression of FXYD1, a modulator of Na(+)K(+)-ATPase activity, was increased, thus favoring puberty onset by maintaining GnRH neuronal excitability (60). Evidence from another mouse model of RTT suggested that regulation of gonadotropin-releasing hormone by *MeCP2* could influence the onset of puberty. For example, in case of *MeCP2* dysfunction (truncating deletion), altered estrogen receptor expression could provide the earlier stimulation of breast development (61).

A recent study by Yang et al. underlined the coexistence of PP and more severe neurological disorders (abnormal EEGs and intractable epilepsy) (35). In this regard, previous studies have shown that epileptic activity, especially mediated through the amygdala, alters reproductive function, including changing ovarian cyclicity in females and altering sex steroid hormone levels in both sexes (62). Different studies demonstrated that *MeCP2* co-3 localizes with GnRH within GnRH neurons in the hypothalamus (63, 64). It seems that *MeCP2* could be a potential player in the regulation of human pubertal timing. In this regard, some authors reported rare heterozygous *MeCP2* mutations in girls with central precious puberty (CPP), with or without neurodevelopmental abnormalities (63–65).

In 2013, Bas et al. reported for the first time a case of central PP in a 6-year-old RTT Turkish girl with *MeCP2* missense mutation (C455G P152R) (33). In 2015 Knight et al. investigated pubertal development in a longitudinal population-based study in RTT. They included 213 female patients born since 1976, using the Australian Rett syndrome database, and reported 6% of cases of precocious puberty, despite delayed menarche in 9%, indicating that the pubertal timing in RTT may be abnormal with anticipated onset but longer duration. These features may be genotype-dependent and could be influenced by malnutrition, growth issues, and bone maturation (32). Likewise, Killian et al. examined individuals with both clinical diagnoses of Rett syndrome or mutations in *MeCP2* using the US Natural History Study database (19). More than 25% of them initiated puberty early yet entered menarche late; only 4% experienced delayed thelarche. This strange trajectory in RTT could be again related to the effect of BMI and genetics. But while BMI was associated with the age of onset of thelarche and pubarche, mutation type was significantly associated with menarche, with the most severe mutation (for example R168X and R255X) predicting later menarche. However, these findings raised additional questions regarding pubertal trajectory in RTT.

Even if previous studies on gonadal and adrenal steroids suggested normal sex hormones in RTT (26), recently there has been growing clinical evidence about the association between *MeCP2* mutations and precocious puberty. In 2019, Bernstein et al. described 2 cases of RTT girls with *MeCP2* deletion together with intellectual disability, obesity, metabolic syndrome, macrocephaly, and precocious puberty. The genetic analysis showed in both a new variant, c.1162_1172del; p.Pro388 not

listed in the Rett database (34). Both patients did not show the full manifestations of Rett syndrome symptoms but presented with intellectual disability and seizures. Moreover, both displayed precocious puberty and obesity.

Recently a multiethnic cohort of 404 patients with idiopathic CPP was tested for genetic study including the research for *MeCP2* mutations by Sanger sequencing (63). It was found that four rare heterozygous *MeCP2* variants (*de novo* missense and insertion), were present in seven girls with CPP. These four *MeCP2* variants identified in CPP girls have not been associated with the Rett syndrome phenotype, except for one case (p.Arg97Cys variant). The other patients presented with different clinic phenotypes, including obesity and autism or microcephaly.

Again, Pepe et. al, in their observational RTT cohort study reported a prevalence of PP of about 15.4%, mainly due to *MeCP2* mutations, higher than other prevalence studies. Of course, the interpretation of this finding should also consider ethnic diversity, in addition to the Western trend to PP recorded in the last decades among healthy girls (23).

4.3.2 Menstrual irregularities

Recent studies revealed that the average age of menarche in females with Rett syndrome was 12.2 years (SDs +/- 5.4 years). However, data are scarce and not univocal. Killian et al. found that 13% of RTT girls reached menarche prematurely compared to the general population, whereas 19% experienced delayed menarche. Menarche occurred earlier in those with a milder mutation genotype (19). Developmental disability often makes menstrual discomfort challenging for caregivers (66), and patients cannot adequately communicate their symptoms and needs. Few studies evaluated specifically this aspect in RTT girls. Hamilton et al. used an anonymous web-based survey for girls 10-25 years old recruited from Rett syndrome LISTSERV in 2009 (36). The results showed that the mean age of menarche was 11.7 years (SD +/- 2.0 years), that the majority of the girls (62%) reported periods of 3-7 days, and 48% of them were using hormonal contraception at the time of the survey; at least one symptom of dysmenorrhea was reported in 76% of cases, with cramps and low back pain the most frequently presented symptoms.

An interesting retrospective cross-sectional chart review and prospective survey in 2020 described data on features of menstruation and menstrual-related symptoms in a large cohort of RTT girls, reporting the prevalence, types, and efficacy of hormonal treatment (37). The most frequent symptoms include dysmenorrhea (61.0%) and emotional lability (49.4%). Features of menstruation in RTT were like those in the general population, except for an increase in catamenial seizure.

Menstrual cycle irregularities were a common finding in the most recent Italian RTT cohort study, accounting for about half of all the endocrinopathies reported in the study population (23), predominantly oligomenorrhea and secondary amenorrhea. Remarkably, the entire group of patients suffered from body weight alterations, both overweight and underweight. Furthermore, premature ovarian failure (POF) was diagnosed in two RTT patients with secondary amenorrhea, enhancing a possible link between RTT and POF. Such association is still almost unknown, even if *FMR1* (Fragile X messenger ribonucleoprotein 1) and *FMR2* genes, often involved in POF with genetic etiology, are located on the long arm of chromosome X (Xq27.3 325 and Xq28, respectively), next to *MeCP2*, and therefore mutations of these genes might explain POF in RTT (67).

4.4 Thyroid disorders

In consideration of documented *MeCP2's* function in maintaining the mature neuronal state, its absence and alterations could have several implications for the balance between synaptic excitation and inhibition (68). The histological findings observed in *MeCP2*-deficient mice especially the loss of parvalbumin neurons in the cerebral cortex share many similarities with that of hypothyroid mice. A typical neuropathological finding caused by thyroid hormone insufficiency is a decrease in the parvalbumin of GABAergic neurons (69). Parvalbumin is a protein expressed in GABAergic neurons in the central nervous system. immature cortical formation in the cortex of *MeCP2* -deficient mice, a phenomenon also observed in hypothyroid mice (70).

In a recent experimental study, human-induced pluripotent stem cells (iPSC) were used to generate *MeCP2* knockout neuronal progenitor cells and adult neurons and then investigate the expression of genes associated with thyroid hormone homeostasis (deiodinases and transporters). *MeCP2* -knockout cells cause alterations in thyroid hormone-related genes, such as hormone transporters and deiodinases (71). In consideration of these results, one hypothesis is that *MeCP2* probably has a significant influence on the assembly of the thyroid system in the body.

Literature data about thyroid disorders in RTT patients are extremely scarce and contrasting. Cooke et al. reported for the first time in 1995 that patients with RTT might have thyroid dysfunction, such as a significant decrease in serum total FT4 concentration. Their study found minimal changes in the TSH levels in RTT patients with no evidence of clinical hypothyroidism. Some of the subjects included with RTT were treated with anticonvulsant drugs. In conclusion, lower thyroid function in RTT was related to the thyroid gland's primary dysfunction and altered hypothalamic-pituitary axis function, considering that even T4-binding proteins were normal (72). Another study by Huppke et al. showed normal age-appropriate plasma values for FT4, TSH, and TSH-night rhythm (26).

Recently, two Italian studies focused on the prevalence of thyroid disorders in RTT. The first one by Stagi et al. (24) reported that FT3 and TSH levels were higher in RTT patients versus controls, even if without reaching statistical significance; moreover, FT4 levels were significantly higher in the RTT group (17.7%), especially in those with *CDKL5* deletions. This finding may support the hypothesis that in many patients with RTT, higher FT4 levels with normal TSH could reflect the attempt to increase and maintain the action of TSH at the central nervous system level, and *MeCP2* is thought to be necessary to stabilize the mature neuronal state (23, 68). Instead, few data are available to understand the molecular connection between *CDKL5* deletions and thyroid dysfunction. The second Italian study by Pepe et al. (23) reported thyroid disorders in approximately 10% of 51 RTT patients, ranging from autoimmune thyroiditis to central

hypothyroidism and hyperthyreotropinemia, with *MeCP2* mutations being the most frequent genotype. Although there is not a clear explanation regarding the behavior of *MeCP2* transcripts and translated products which differs between organs, it seems that thyroid hormones may have a pivotal role in the expression of *MeCP2* and the relationship between thyroid function and *MeCP2* might be that of reciprocal interactions rather than one-way interactions (73).

4.5 Bone Health and Orthopedic Issues

Evidence from both human and animal studies supported the hypothesis that *MeCP2* mutations could be associated with altered epigenetic regulation of bone-related factors and signaling pathways, including RANKL/RANK/OPG system. Nevertheless, further studies are needed to better understand the role of *MeCP2* in bone homeostasis (74).

Bone health concerns are relevant in RTT due to motor impairment and skeletal abnormalities. Indeed, it was among the first endocrinopathies described in RTT patients (75, 76). Female patients with RTT often suffer from altered bone health with decreased mineral content, decreased mineral density, and an increased fracture rate three to four times that of normal females. Common findings are orthopedic problems including scoliosis and joint contractures (76). Scoliosis is related to the lack of walking action, whereas it seems to be unrelated to the loss of hand skills or hand stereotypes. Several markers, such as osteocalcin or bone-specific alkaline phosphatase, were found to be reduced in RTT, thus enhancing low bone turnover in these patients (77). Such alteration of bone mineral deposition may be caused by vitamin D deficiency typical of RTT population, together with the lack of spontaneous mobilization (41).

Budden et al. studied osteopenia and osteoporosis in RTT girls analyzing bone remodeling by quantitative bone histomorphometry. They found a slow rate of bone formation, thus negatively influencing development and accumulation of peak bone mass and contributing to decreased bone volume in Rett syndrome (40). In the same direction, Motil ed al. underlined that decreased bone formation, rather than increased bone resorption, may partially explain the deficits in bone mineral mass in RTT (low bone mineral content 59%, low bone mineral density 45%) and that dietary factors, but not hormonal or inflammatory markers, were associated with altered bone mineral status. This requires adequate calcium, protein, and phosphate diet assumptions, to improve bone health in RTT. Osteocalcin concentrations for all age groups were significantly lower in RTT study population, whereas the concentrations of bone alkaline phosphatase were significantly higher (38).

Even if the normal ghrelin/GH/IGF-1 axis stimulates longitudinal bone growth, Caffarelli et al. reported that plasma levels of ghrelin did not reflect longitudinal bone growth in female RTT patients within a growing period (78).

Jefferson et al. used densitometry (DXA) to evaluate bone density and content, analyzing how factors such as genotype, epilepsy, BMI, and mobility might affect these parameters. They confirmed low bone mineral density and bone mineral content in RTT, particularly at the femoral neck DXA. This finding seemed to be directly associated with the type of *MeCP2* mutation, especially p.R168X and p.T158M (39). Indeed, the lack of *MeCP2* may reduce bone density through osteoblastic dysfunction (79).

Shapiro et al. reported bone mineral density values 2 SD below age-related norms in 48.9% of RTT patients (42). Motil et al. observed low bone mineral content or fractures in 37% of RTT patients, with older RTT females showing more severe phenotypes rather than younger ones (31).

Finally, the prevalence of osteopenia and osteoporosis reported in the observational study by Pepe et al. is lower than the previous study (19.6%), probably because of infrequent testing of bone status outside the research setting or particular clinical situations (23).

The last clinical guideline for the management of bone health issues in RTT highlighted the need for both fracture and low bone densitometry for a diagnosis of osteoporosis in RTT. Increasing physical activity and initiating calcium and vitamin D supplementation is recommended in Rett patients (74, 80).

5 Limitations of the study

The main limitation of this review is the heterogeneity of outcomes and design of the studies included. The majority of them were not focused on the primary aim of the endocrinopathies in RTT. The Rett population included in this review was not homogenous in terms of sex, age, ethnicity, country, and genotype. However, this feature could be seen as a point of value of the review, as it collected multifaceted aspects of the same genetic syndrome. The number of patients included was often small, except for studies based on registers. Only a few studies recruited male RTT population and genotype other than *MeCP2* mutations.

Nevertheless, a common aspect of the studies selected was the attention to the type of mutation in *MeCP2*, the prevalence of endocrinopathies, and their severity, in addition to the focus on genotype-phenotype correlation especially in the severe Rett forms and mutations. Of course, systematic data are needed to confirm these findings.

Finally, the features of associated epileptic activity in RTT (type and severity, age of onset, control of disease, drugs), were not constantly reported in the studies available, even if it is known to potentially influence endocrine function.

Despite some limitations, to the best of our knowledge, this is the first systematic review specifically focused on reporting the prevalence of endocrinopathies in RTT. The results showed that endocrine disorders represent a common finding in RTT, and therefore should be adequately investigated, to improve the quality of life and the care of these patients. Due to the lack of specific recommendations, we hope that our effort could highlight the need of periodic endocrinological follow-up, to prevent and detect endocrinological comorbidities at an early stage. A proposal for RTT patient's endocrinological management is synthesized in Table 2.

6 Conclusion

Rett syndrome is a severe neurological disorder that has increasingly emerged in recent years. Multiple organs and apparatus can be involved, with a broad spectrum of manifestations. The present systematic review shows that endocrinopathies are not rare in RTT patients, with malnutrition, short stature, pubertal abnormalities, and bone disorders being the most frequent endocrinological findings. Interestingly, patients with *MeCP2* alterations seem at higher risk for developing endocrinopathies because of this protein's ubiquitous

TABLE 2 Proposal for endocrinological screening and follow-up in Rett syndrome, from diagnosis to adulthood.

ENDOCRINOLOGICAL MANAGEMENT	INFANCY AND CHILDHOOD	ADOLESCENCE	ADULTHOOD
Growth evaluation			
Linear growth	√	√	NA
Weight gain and BMI	√	√	√
Tanner stage	√	√	NA
IGF-1 and GH secretion	*in case of short stature and/or growth deceleration	*in case of short stature and/or growth deceleration	NA
Bone age	√	*in case of short stature and/or growth deceleration	NA
Thyroid function			
FT4, TSH	√	√	√
TPO-AB, TG-AB	√	√	√
Thyroid ultrasound	√	√	*in case of thyroid disorders, goitre or family history
Metabolic assessment			
Blood pressure	√	√	√
Waist circumference	*if BMI > 2.0 SDS	*if BMI > 2.0 SDS	*if BMI > 2.0 SDS
Blood glucose	√	√	√
Insulin, OGTT, Hba1c	*if BMI > 2.0 SDS	*if BMI > 2.0 SDS	*if BMI > 2.0 SDS
Lipid profile	√	√	√
Gonadal function			
LH, FSH, PG, E2	*in case of clinical signs of precocious puberty	*in case of delayed puberty or menstrual irregularities	*in case of menstrual irregularities
LHRH test	*in case of clinical signs of precocious puberty	*in case of pubertal alterations	NA
AMH	NA	*in case of amenorrhea	*in case of amenorrhea
FSH, LH, T, AMH, INIBIN B	NA	*in case of male disorders of pubertal development	*in case of male disorders of pubertal development
Pelvic ultrasound	*in case of clinical signs of precocious puberty	*in case of delayed puberty or menstrual irregularities	*in case of menstrual irregularities
Bone health			
Calcium-phosphorus metabolism	√	√	√
DEXA	*if necessary	Every 2 years	Every 2 years
25-OH vitamin D supplementation	√	√	√
Other			
Nutritional counselling/support	√	√	√

*NA, Not applicable.
BMI, body mass index; SDS, standard deviation score; DEXA, dual-energy X-ray absorptiometry.
√, to be performed at diagnosis and periodically thereafter.

distribution. These data highlight the need to recommend a specific endocrinological evaluation and follow-up in all RTT patients, to ensure a better quality of life in a multidisciplinary approach.

Data availability statement

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

Author contributions

GP: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. RC: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. DC: Writing – review & editing, Supervision, Project administration, Formal analysis. GL: Writing – review & editing, Supervision, Project administration, Formal analysis. LM: Writing – review & editing, Visualization, Funding acquisition, Formal analysis. CL: Writing – review & editing, Visualization, Investigation, Funding acquisition. TAb: Writing – review & editing, Visualization, Project administration, Data curation. GZ: Writing – review & editing, Supervision, Software, Funding acquisition. TAv: Writing – review & editing, Supervision, Project administration, Formal analysis, Data curation. SS: Writing – review & editing, Software, Investigation. MW: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Conceptualization.

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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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Relationship between liver and cardiometabolic health in type 1 diabetes

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Introduction: Type 1 diabetes (T1D) is a chronic condition marked by insulin deficiency and hyperglycemia, with an increasing global incidence, particularly among children. Despite improvements in diabetes management, individuals with T1D continue to experience higher rates of cardiovascular disease (CVD), the leading cause of mortality in this population. Traditional CVD risk factors such as dyslipidemia and poor glycemic control are insufficient to fully explain the elevated risk in T1D, prompting further investigation into additional factors. Emerging evidence suggests that metabolic dysfunction-associated steatotic liver disease (MASLD) plays a critical role in this heightened CVD risk.

Objective: This narrative review aims to explore the relationship between MASLD and CVD in individuals with T1D. The review focuses on the prevalence of MASLD, its contributing risk factors, and the potential impact of liver dysfunction on cardiovascular outcomes in this population.

Methods: A review of existing literature was conducted, focusing on observational studies, cohort studies, and meta-analyses that investigate the prevalence of MASLD in T1D populations and its association with CVD. The review also examines the physiological mechanisms linking MASLD and CVD, including insulin resistance, systemic inflammation, and hepatic dyslipidemia. Key studies were evaluated to identify patterns in MASLD prevalence based on diagnostic modalities and to assess the independent contribution of MASLD to cardiovascular risk in T1D patients.

Conclusion: MASLD is increasingly recognized as a significant contributor to CVD in individuals with T1D, particularly in those with shared risk factors like obesity and insulin resistance. Evidence suggests that MASLD exacerbates hepatic and systemic metabolic dysfunction, increasing CVD risk through mechanisms such as chronic inflammation and atherogenic lipid profiles. Routine liver health assessments and tailored management strategies targeting MASLD should be incorporated into clinical care for individuals with T1D to mitigate long-term cardiovascular complications.

KEYWORDS

hepatosteatosis, cardiovascular disease, insulin resistance, inflammation, type 1 diabetes

1 Introduction

Type 1 diabetes (T1D) is a common chronic metabolic disorder characterized by insulin deficiency and hyperglycemia, with an estimated worldwide prevalence of 95 per 100,000 people (1). Globally, the incidence of T1D is rising, particularly among children, although an estimated 25-50% of new diagnoses occur in adults (2–5). Managing T1D poses significant health challenges due to the chronic and highly demanding nature of the disease. Despite meticulous management, individuals with T1D remain at risk of developing both acute and long-term complications.

Recent advances in treatment, particularly hybrid closed-loop systems, have substantially improved quality of life and reduced the incidence of insulin-related acute complications such as severe hypoglycemia, hyperglycemia, and ketoacidosis (6). However, despite significant advances in diabetes management and recommendations of aggressive strategies to control cardiometabolic risk factors (i.e., lower blood pressure and cholesterol thresholds), individuals with T1D continue to experience higher rates of morbidity and mortality compared to the general population (7). Cardiovascular diseases (CVD) remain the leading cause of death among people with T1D. While traditional risk factors such as dyslipidemia, hypertension, and poor glycemic control play a role, they do not fully explain the increased risk of adverse CVD outcomes. This underscores the need for further research to identify additional contributing factors (8).

Recent studies suggest a link between metabolic dysfunction–associated steatotic liver disease (MASLD) and CVD In patients with T1D. In this narrative review, we will explore risk factors for CVD, the emerging role of the liver in cardiometabolic health, and management of MASLD and CVD risk in individuals with T1D. A deeper understanding of MASLD's impact on CVD in T1D could lead to more accurate risk assessments and improved prevention strategies.

2 Cardiovascular disease in type 1 diabetes

Cardiovascular disease is highly prevalent among individuals with T1D and is the leading cause of morbidity and mortality in this population (7). Coronary artery disease/atherosclerosis is the most common type of CVD (9). However, peripheral artery disease, cerebrovascular disease (stroke), heart failure, and cardiomyopathy also occur more frequently in those with T1D compared to the general population.

The increased burden of CVD in individuals with T1D has been consistently reported in observational studies across various cohorts. Earlier studies in individuals with juvenile diabetes (diagnosed before age 21 years) reported higher cumulative mortality rate (35 ± 5%) due to coronary artery disease compared to healthy subjects in the Framingham Heart Study (8% for men and 4% for women) by age 55 (10). Similarly, the Pittsburgh Epidemiology of Diabetes Complications study of childhood-onset type 1 diabetes showed a 19-fold increased relative risk of CVD mortality particularly in younger adults (< 45 years) compared to the age-matched background population (11). More recently, in a large nationwide cohort study in Denmark involving young people (ages 1–39), CVD mortality was found to be 11 times higher in individuals with T1D compared to age-matched individuals without diabetes (12).

Among adults with T1D, the prevalence of CVD is seven times higher than in healthy controls and typically arises a decade earlier, resulting in a shortened life expectancy by an average of 10 years (7, 13). This was further supported by a Swedish cohort study, which demonstrated that the age at T1D diagnosis is an important determinant of CVD risk. Individuals diagnosed between ages 0–10 had a fivefold higher risk of developing CVD compared to those diagnosed between ages 26–30 (14). Additionally, early-onset T1D was associated with a greater loss of life expectancy compared to late-onset T1D, with CVD-related mortality being higher among women than men (14).

Although clinically manifest CVD is rare before adulthood, early signs – such as increased arterial intima-media thickness (IMT), arterial stiffness, and decreased pulse wave velocity (PWV) – can be detected during childhood. These findings are not surprising, as CVD risk factors are prevalent among youth with T1D. In a cohort study involving multi-ethnic children with T1D in the UK, up to 60% of patients were found to have at least one traditional CVD risk factor (15). Several studies, including SEARCH-CVD (an ancillary study to SEARCH for Diabetes in Youth) in the US, have shown increased carotid intima-media thickness (cIMT) in youth with T1D compared to healthy controls (16). A systematic review and meta-analysis by Giannopoulou et al., which compiled data from 23 studies (n=2,860 T1D and n=1,861 control subjects), found higher cIMT and PWV in children with T1D than in matched controls (17).

3 CVD risk factors in individuals with diabetes

The increased prevalence of CVD in individuals with diabetes is attributed to a combination of modifiable and non-modifiable factors, further complicated by social determinants of health and individual-level differences in behaviors (18). Prolonged hyperglycemia, dyslipidemia, hypertension, smoking, obesity, and presence of microvascular complications (i.e., nephropathy) are regarded as traditional risk factors for adverse CVD outcomes (19). While improvements in these risk factors are associated with reductions in cardiovascular events and decreased mortality, even sustained improvements do not fully mitigate adverse CVD outcomes. This suggests the role of additional unexplored risk factors in CVD morbidity and mortality in T1D (20). Recently, emerging roles of insulin resistance (IR), visceral adiposity, chronic inflammation, increased oxidative stress, epigenetic modification, and cardiac autoimmunity have been recognized in the pathogenesis of CVD in diabetes (21, 22).

4 The link between metabolic dysfunction-associated steatotic liver disease, CVD, and diabetes

4.1 MASLD and diabetes

Metabolic dysfunction–associated steatotic liver disease (MASLD), formerly known as nonalcoholic fatty liver disease or NAFLD, is the most common etiology of chronic liver disease in children and adults (23, 24). The MASLD spectrum ranges from simple hepatosteatosis (HS) to metabolic dysfunction–associated steatohepatitis (MASH), cirrhosis, and eventually liver failure. MASLD is considered the hepatic manifestation of metabolic syndrome and is strongly associated with obesity, insulin resistance, dyslipidemia, hypertension, and increased waist circumference. It is estimated that up to 10% of all children in the United States have some form of steatotic liver disease, with a prevalence of up to 50% among girls with obesity and polycystic ovarian syndrome (PCOS) (23, 25). MASH-related cirrhosis is one of the leading causes of liver transplantation (25). Despite the well-established relationship between MASLD and T2D, the association between MASLD and T1D has been largely unexplored.

Rates of obesity among individuals with T1D are increasing in parallel with global population trends in both children and adults, with more than a third of patients with T1D estimated to have overweight or obesity (26, 27). Excess weight gain may also occur over time due to intensive insulin therapy, inadequate exercise, and excess food intake, often in defense against hypoglycemia. Despite a substantial proportion of T1D subjects sharing similar phenotypes to those with T2D, such as obesity, insulin resistance, dyslipidemia, chronic hyperglycemia, and systemic hyperinsulinemia, routine screening for MASLD in T1D is not the standard of care in clinical practice. However, ADA's 2024 standards of care guidelines urge the providers to consider screening for fibrosis in people with T1D in the presence of additional risk factors for MASLD such as obesity, incidental hepatic steatosis on imaging, or elevated plasma aminotransferases.

4.2 Prevalence of MASLD in T1D

Epidemiological studies assessing the prevalence of MASLD in youth with T1D are scarce. Furthermore, results from the available studies are not readily comparable due to differences in the diagnostic modalities and study populations (Table 1). In a meta-analysis assessing MASLD prevalence in T1D, comprising 20 studies in children and adults, De Vries and colleagues (28) found a pooled prevalence of 7.9% (95% CI: 2.6-15.5%) among children with T1D (n=202 in 3 studies), compared to 22.0% (95% CI: 13.9-31.2%) among adults with T1D (n=3699 in 17 studies).

MASLD prevalence has consistently been reported as higher in studies utilizing ultrasound as the diagnostic tool (29–33). This may be partly due to ultrasound's inability to distinguish glycogenopathy and fatty infiltration in liver images. For example, Regnell et al. (34) found no cases of MASLD using hepatic fat fraction MRI for diagnosis in a small set of children (n=22) with T1D. Similarly, Kummer et al. (35) reported only one potential MASLD case in a cohort of 93 children using a combination of ultrasound, transient elastography (FibroScan®), and acoustic radiation force imaging. However, these studies had very few or no subjects with obesity, limiting their generalizability given the rising prevalence of obesity among children with T1D. In contrast, a study using MRI as the diagnostic tool linked MASLD to obesity (36). Likewise, in our previous work, we demonstrated that pediatric patients with T1D and obesity (n=15) shared similar clinical, laboratory, and imaging findings (FibroScan®) compared to patients with obesity without T1D (n=28), supporting the notion that obesity is the major determinant of MASLD (37). In a cross-sectional study, West et al. (38) demonstrated that elevated serum alanine aminotransferase (ALT) levels were more common in T1D patients (n=517, majority adults) than in the general population, with a prevalence of 9.5% (95%CI: 7.1-12.3%).

More recently, Koutny et al., using serum ALT levels as a proxy for MASLD, examined the longitudinal data from 32,325 children (age 2-17 years) with T1D in the Diabetes-Patienten-Verlaufsdokumentation study (39). They reported that children with poorly controlled T1D (i.e., HbA1c > 11%) had 2.54 (95% CI: 2.10-3.10) times the odds of having elevated liver enzymes after adjusting for sex, age, diabetes duration, and overweight status. Their findings suggest that the association between T1D and MASLD is independent of weight status.

Estimates of MASLD burden among T1D patients vary widely, depending on the population studied and diagnostic modality used, leaving the true prevalence and impact of MASLD in T1D remain unclear (40). Moreover, there is a paucity of pediatric studies in the T1D population utilizing histology (i.e., liver biopsy) as the diagnostic tool. As a result, the prognostic importance of elevated liver enzymes or abnormal imaging findings remains unknown. In a more recent meta-analysis, Ciardullo and Perseghin reported a 5% prevalence for increased liver stiffness, a surrogate marker for fibrosis, in adult subjects with T1D (41).

4.3 Factors contributing to MASLD development in T1D

The "two-hit" model and its extension, the "multiple parallel hits" hypothesis, offer a detailed understanding of the pathogenesis of MASLD and its systemic repercussions. According to these frameworks, chronic inflammation in MASLD emerges from a combination of various stressors acting simultaneously, such as insulin resistance and lipotoxicity, particularly in genetically susceptible individuals (42, 43). Excessive liver fat accumulation in MASLD results from an imbalance between lipid accumulation and utilization. This imbalance is driven by peripheral and hepatic IR, which promotes increased hepatic uptake of free fatty acids and enhanced triglyceride synthesis. When the liver's ability to oxidize fatty acids or export triglycerides as very-low-density lipoproteins cannot keep pace with fat accumulation, hepatosteatosis occurs.

In T1D, several factors contribute to MASLD, mirroring those seen in obesity and T2D. These factors include poor glycemic

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TABLE 1 MASLD prevalence studies in pediatric T1D patients.

Reference (#)	N	Sex (Male %)	Age (years)	Diabetes Duration (years)	HbA1c (%)	Diagnostic Tool for MASLD	MASLD Prevalence (%)	Key features of the study
Al-Hussaini (29)	106	42	8.5 ± 2.8	2.2 ± 2.1	10.7 ± 2.4	Ultrasound	21	Half of the patients with abnormal US had hepatomegaly
El-Karaksy (30)	692	48	9.7 ± 4.2	6.9 ± 3.7	7.7 ± 1.6	Hepatomegaly on exam, elevated liver enzymes, ultrasound	8.7	Patients with viral hepatitis (n=25) were included in the analysis.
Elkabbany (31)	100	39	13.7 ± 1.9	6.5 ± 1.7	9.7 ± 2.4	Hepatomegaly on exam, elevated liver enzymes, ultrasound, transient elastrogram	12	Patients with autoimmune hepatitis and viral hepatitis were included in the analysis but MASLD prevalence given separately.
Aydin (32)	110	47	12.5 ± 2.8	3.1 ± 2.2	11.4 ± 2.1	Ultrasound	15.5	Patients with abnormal US had HbA1c >9%
Abdallah (33)	74	50	14.3 ± 3.0	6.3 ± 3.0	10.3 ± 2.0	Ultrasound	62	Only 6.5% of patients with abnormal US had elevated liver enzymes.
Regnell (34)	22	54	13.5 (range: 9-17)	5.9 (range: 0–13)	7.9 (range: 6.4–11.1)	MRI	0	There were none to a few overweight or obese subjects in this cohort.
Kummer (35)	93	49	11.9 ± 4.0	4.6 ± 3.5	7.6 ± 0.8	Liver Enzyme, Ultrasound,	10.8	Only two patients had obesity and only 1 had ALT level twice the upper limit of normal.
Sae-Wong (36)	50	44	16.9 (IQR: 13.6-20)	6.5 (IQR: 4-11)	8.7 (IQR: 7.9-10.1)	MRI	10	Of the 5 patients with MASLD, 2 were overweight and 2 were obese.
Tas (37)	49	53	14.5 ± 3	4.9 ± 3.6	8.7 ± 2	Transient elastrogram	33	A CAP >241 dB/m was used for MASLD diagnosis. 30% of the T1D patients had obesity.
Koutny (39)	32,325	53	Average age across strata ranges between 11.3 and 15 yrs	Average across strata ranges between 1.6 and 4.7 years	Groups stratified per HbA1c (<9%, 9-11%, >11%)	Liver enzyme	14.4	For male participants, ALT levels > 26 U/L and for female participants> 22 U/L were considered elevated

control, obesity, IR, dyslipidemia, lipoprotein abnormalities, poor dietary habits, impaired systemic to portal insulin gradient, and altered gut microbiome in genetically susceptible individuals (44, 45).

In a cross-sectional study of young adults with T1D (n=659, mean age 37 ± 13 years, mean diabetes duration 20 ± 12 years), Della Pepa et al. (46) reported an association between elevated HbA1c and MASLD. In their cohort, patients with HbA1c > 7.6% had significantly higher liver indices—the fatty liver index (FLI) and hepatic steatosis index, two commonly used non-invasive composite scoring systems to determine risk for MASLD with higher scores predicting MASLD. This association was independent of obesity status. A more recent cross-sectional study examined the association of continuous glucose meter-derived outcomes with MASLD in adults with T1D (n=302, median age 49 [34-61] years, median diabetes duration 29 [17-38] years).The study found independent associations between MASLD (ultrasound diagnosed) and time in range (55% ± 16%, p=0.028), time above range (p=0.007), and time below range (p=0.036) (47). These studies indicate that overall glycemic control and blood glucose variability are linked to MASLD.

As mentioned above, individuals with T1D often have obesity rates comparable to their age-matched peers at diagnosis and are at risk for further weight gain due to systemic insulin therapy, frequent snacking to manage hypoglycemia, and reduced physical activity (48–50). The interplay between hyperglycemia, hyperinsulinemia, and hyperglucagonemia exacerbates IR. Elevated glucose levels enhance hepatic glucose uptake through glucose transporter 2 (GLUT 2) and stimulate genes encoding sterol regulatory element-binding proteins (SREBP) and carbohydrate-responsive element-binding protein (ChREBP), two transcription factors upregulating hepatic *de novo* lipogenesis (51). Additionally, systemic hyperinsulinemia and reduced hepatic insulin clearance —potentially due to decreased expression of carcinoembryonic

antigen-related cell adhesion molecule 1 (CEACAM1) because of hepatic IR—further promote hepatic lipogenesis (52).

These mechanisms collectively contribute to the development of MASLD in T1D patients. Moreover, steatotic or inflamed liver releases pro-inflammatory and pro-coagulant factors mediating vascular endothelial damage (53).

4.4 Does MASLD increase CVD risk?

The link between MASLD and adverse CVD outcomes is well-documented in subjects with obesity and T2D (54). Examining the nationwide health screening database of more than 9.5 million adults (age 40-64 years) in South Korea, Lee et al. (55) estimated a hazard ratio of 1.43 (95% CI: 1.41-1.45) for CVD events – such as myocardial infarction, ischemic stroke, heart failure, or CVD-related death – in individuals with MASLD compared to a reference group with no fatty liver disease (unadjusted for diabetes or other potential mediators). Similarly, Vaz et al. (56) analyzed the data obtained longitudinally from adults in Australia (25,469 person-years follow-up) and found that subjects with MASLD had a 1.5 times (95% CI: 1.11-2.06) the hazard ratio of adverse CVD outcomes, including non-fatal myocardial infarction, cerebrovascular accidents, and CVD-related deaths, after adjusting for demographic covariates and known cardiometabolic risk factors, including diabetes. Both studies used fatty liver index, a biomarker panel for MASLD diagnosis.

The relationship between MASLD and CVD is hypothesized to be bidirectional such that MASLD is not only a marker of CVD but also a contributor to its pathogenesis. Hepatosteatosis might contribute to hepatic and systemic IR, and atherogenic dyslipidemia (57). Steatosis triggers inflammatory pathways and cytokine release, leading to liver fibrosis and systemic inflammation. Studies highlight the predictive role highly sensitive C-reactive protein as a marker of low-grade systemic inflammation in adverse CVD outcomes in patients with MASLD (58, 59). However, due to overlapping risk factors for the development of CVD and MASLD, it remains challenging to assess a causal relationship (Table 2).

Emerging evidence suggests a connection between MASLD and CVD in adolescents and young adults. A Swedish nationwide cohort study (60) found that children and young adults under 25 years (n=699) with biopsy-confirmed MASLD had significantly higher rates of incident CVD events compared to matched population controls. Specifically, they reported adjusted hazard ratios (aHR) of 2.33 (95% CI: 1.43 to 3.78) for ischemic heart disease, 3.07 (95% CI: 1.62 to 5.83) for congestive heart disease, and 3.16 (95% CI: 1.49 to 6.68) for arrhythmia after a median follow-up of 16.6 years. Notably, the incident rates were higher in the advanced liver disease subgroup, suggesting a possible role of the liver in the progression of CVD. Additionally, a recent systematic review and meta-analysis (61) that examined the data from 4 large cohorts (10,668,189 participants) corroborated these findings, demonstrating a strong link between MASLD in young adults and children and the risk of CVD, with a HR of 1.63 (95% CI: 1.46–1.82, p<0.001).

TABLE 2 Risk factors for CVD and MASLD in type 1 diabetes and diagnostic tools.

Traditional Risk Factors	Emerging Risk Factors
Obesity	Chronic inflammation
Hyperglycemia (i.e., poor glycemic control)	Oxidative stress
Dyslipidemia	Gut microbiota
Hypertension	Environmental factors
Smoking	Cardiac autoimmunity[$]
Lifestyle factors (unhealthy diet, limited physical activity)	Microvascular complications of diabetes
Insulin resistance	[$]Unique to CVD.
Visceral adiposity (i.e., increased waist circumference)	
Screening/Diagnosis of MASLD	
Physical examination (i.e., hepatomegaly*)	
Increased liver enzymes (ALT, AST, GGT)^,*	
Ultrasound (B-mode)^	
Vibration controlled transient elastogram (i.e., Fibroscan®)	
Magnetic resonance elastogram (i.e., spectroscopy, PDFF) [#]	
Liver biopsy[##]	

* Could indicate hepatic glycogenopathy.
^ Low specificity in early stages/low degree of hepatosteatosis.
[#] Non-invasive reference standard tool.
[##] Gold Standard. Allows histological grading.
[$] refers that "cardiac immunity" is a unique risk factor for CVD and it does not apply to MASLD.

4.5 MASLD and CVD in patients with T1D

Data on CVD and MASLD in T1D population is scarce. Major barriers to directly assessing this relationship, particularly in children and young adults, include the natural course of MASLD and CVD (i.e., long asymptomatic phases), the lack of non-invasive diagnostic tools to stage MASLD, the reliance on composite scores rather than clinical outcomes in CVD assessment, and the presence of confounding factors that often co-exist with both conditions such as dyslipidemia, hypertension, visceral adiposity, and obesity.

Despite these challenges, a few studies have shown that MASLD is independently associated with adverse cardiovascular outcomes (62) and microvascular complications (63, 64) in individuals with T1D. In an observational study of 286 adults with T1D (median diabetes duration 17 [10-30] years), Mantovani et al. (62) assessed baseline MASLD status via ultrasound and followed the patients for a mean of 5.3 ± 2.1 years for the occurrence of incident CVD events. After adjusting for demographic and clinical covariates, the incidence of composite CVD events was found to be 6.73 times (95% CI: 1.2–38.1) higher in those with MASLD than those without MASLD, highlighting the importance of non-traditional risk factors for adverse CVD outcomes. The same research group replicated their findings on two other cohorts, demonstrated an independent relationship between MASLD and CVD, even after adjusting for age, sex, body mass index, glycemic control (i.e., HbA1c), diabetes duration, plasma lipids, albuminuria, smoking status, and family

history of CVD (65, 66). It is important to note that the presence of fatty liver was assessed using ultrasound, and the prevalence of MASLD varied between 44 and 52% in these studies, which is significantly higher than most other prevalence studies (28).

Cross-sectional studies from two different research groups reported similar findings. Zhang et al. (67) assessed the relationship between MASLD and cIMT in adult T1D patients. Of the 722 patients (mean age 46 ± 13 years, diabetes duration 7.5 ± 4.2 years), 15.9% had MASLD by ultrasound. This subgroup had greater cIMT (0.81 ± 0.25 vs. 0.69 ± 0.18 mm; p<0.001) than non-MASLD patients even after adjusting for potential confounders. Moreover, MASLD was independently associated with cIMT in a linear regression model (standardized β, 0.151, p < 0.001). Similarly, Serra-Planas et al. (68) examined the prevalence of MASLD and its relationship to CVD risk factors in 100 adult T1D patients (mean age 39.4 ± 7.8 years, diabetes duration 21.7 ± 8.6 years. They reported a lower MASLD (only 12% of the cohort) by ultrasound. Those with MASLD had a greater cIMT than those without NAFLD (0.65 ± 0.17 vs 0.55 ± 0.14 mm; p=0.029), but the groups did not differ regarding carotid artery calcification score or the presence of carotid plaque—two other markers of CVD.

In a recently published observational prospective study with a median follow-up of 11 years, Garofolo et al. (69) examined the association between MASLD (assessed by FLI), all-cause mortality, and first cardiovascular events in 774 young adult T1D patients (mean age 30.3 ± 11.1 years, diabetes duration 18.5 ± 11.6 years, HbA1c 7.8 ± 1.2%). They showed an increased incident of CVD events in those with elevated FLI, even after adjusting for clinical and biochemical factors considered important in Steno Type 1 and EURODIAB Risk Engines for determining CVD risk.

Traditionally, CVD risk assessment in T1D has focused on factors like glycemic control, blood pressure, lipid levels, and prevention and treatment of diabetic kidney disease. Although the aforementioned studies show an independent association between MASLD and CVD in T1D population, evidence providing a causal relationship is lacking. Given the accumulating data suggesting that MASLD is an independent CVD risk factor, the liver health assessments should be included in risk estimation and individualized treatment.

In an ongoing clinical trial in adults at the University Hospital, Antwerp, Belgium (NCT04664036), the researchers are examining the utility of non-invasive diagnostic tools for assessing MASLD and their correlation with microvascular and macrovascular complications of diabetes in a prospective manner. The findings of study are expected to shed light on liver's role in the development of CVD in individuals with T1D. Detecting and managing MASLD could potentially reduce the risk of developing cardiovascular complications, the leading cause of morbidity and mortality in individuals with T1D.

5 Conclusions

In conclusion, the complex interplay between MASLD and CVD in T1D patients underscores the importance of early detection and comprehensive management of MASLD. Despite advancements in T1D therapies, including continuous glucose monitors and hybrid closed-loop systems, individuals with T1D continue to face an elevated risk of CVD, which is not fully explained by traditional factors such as dyslipidemia, hypertension, and poor glycemic control. Emerging evidence now points to MASLD as a significant contributor to cardiovascular risk, even in the T1D population. MASLD, once primarily linked to T2D, is increasingly recognized in T1D due to overlapping risk factors such as obesity, insulin resistance, and chronic inflammation. This bidirectional relationship between MASLD and CVD, characterized by hepatic insulin resistance, atherogenic dyslipidemia, and systemic inflammation, suggests that MASLD may be both a marker and a mediator of CVD risk in T1D.

Considering available and proposed treatment options, managing MASLD in T1D patients requires a multifaceted approach. Lifestyle interventions, including weight loss, exercise, and dietary modifications, remain central to MASLD management, particularly in individuals with concurrent obesity. Pharmacological therapies targeting the liver are also being explored. Current treatments under investigation for MASLD in T2D, such as GLP-1 receptor agonists (70) and SGLT-2 inhibitors (71), have shown promise in improving liver steatosis and reducing CVD risk and may hold potential for T1D patients. These agents not only improve glycemic control but also promote weight loss, reduce inflammation, and improve lipid profiles —key factors in managing MASLD. Additionally, new therapies targeting fibrosis, such as FGF21 analogs, are in clinical trials and may offer benefits in preventing progression from simple steatosis to more advanced liver disease (72). While no specific treatments have been validated for MASLD in T1D, ongoing research in T2D could inform future therapeutic strategies. Early liver health assessments and tailored interventions in T1D patients could significantly reduce the burden of CVD, emphasizing the importance of integrated cardiometabolic care for improving long-term outcomes.

Author contributions

ET: Funding acquisition, Conceptualization, Data curation, Writing – original draft. BKV: Conceptualization, Writing – review & editing. BM: Writing – review & editing. IL: Conceptualization, Supervision, Writing – review & editing. RM: Conceptualization, Data curation, Funding acquisition, Methodology, Writing – review & editing.

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Conflict of interest

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Long term effects of aromatase inhibitor treatment in patients with aromatase excess syndrome

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Introduction: Aromatase excess syndrome (AEXS) is a rare, autosomal dominant disorder, characterized by enhanced aromatization of androgens and estrogen excess. In males it is characterized by pre-/peripubertal gynecomastia, hypogonadotropic hypogonadism, advanced bone age and short adult height. Only a few female patients have been described so far.

Methods: We report on a family with four members with AEXS and present the long-term effects of aromatase inhibitor use in three of them. Genetic analysis showed a monoallelic 0.3-Mb deletion in 15q21, involving parts of *CYP19A1*, *GLDN* and *DMXL2* in all four patients with AEXS.

Results: The index patient (male, 8 years old) presented with gynecomastia and accelerated growth and bone age. With start of puberty, estradiol levels increased, while testosterone levels remained low. Gynecomastia progressed and a mastectomy was performed twice. Presuming AEXS, a therapy with letrozole was initiated at the age of 19 years. Low-dose letrozole treatment was associated with an increase in testicular volume, increase in virilization and improvement in physical strength and libido. His brother (age 3 years) presented with accelerated growth and bone age. Treatment with letrozole, which was started at the age of 7 years, resulted in achieving an adult height of 179 cm and prevented the appearance of gynecomastia. His sister (age 6 years), who presented with premature thelarche and accelerated growth and bone age, was treated with an estrogen receptor modulator and a GnRH analog followed by letrozole treatment. Menarche occurred at age 13.5 years and adult height was 158 cm. Their father had an early, accelerated growth with an adult height of 171 cm, a delayed puberty and no gynecomastia. *In vitro* studies provided evidence for involvement of aromatase induction in atypical cells and an increased range of potential mechanisms regulating aromatase activity due to the presence of the mutated allele.

Discussion: In conclusion, we observed a phenotypic variability within family members with AEXS carrying the same *CYP19A1* microdeletion. When started

early, treatment with letrozole was found to prevent the development of gynecomastia and increase adult height in one patient. In adult life, low-dose letrozole treatment resulted in improved physical strength and libido in the index patient.

KEYWORDS

gynecomastia, estradiol, aromatase, letrozole, testosterone

Introduction

Aromatase excess syndrome (AEXS, OMIM no. 139300), formerly known as familial gynecomastia, is a rare, autosomal dominant disorder, characterized by enhanced extraglandular aromatization of androgens and estrogen excess (1). AEXS is found to be caused by heterozygous genomic rearrangements in chromosome 15q21.2, causing overexpression of the aromatase gene *CYP19A1* (1, 2). Aromatase is expressed in many tissues and catalyzes the aromatization of the A-ring of androstenedione to produce estrone and the A-ring of testosterone to produce estradiol (3). The prototypical sites of aromatization are the female gonads (ovaries) and the placenta, however, aromatase activity can also be found in other tissues, such as the breast, brain, fetal liver, muscle, bone, testis, skin and adipose tissue, which is the major site of estrogen synthesis in postmenopausal women and in men (4). As so, aromatase is a significant regulator for the balance between estrogens and androgens in both sexes and plays a pivotal role in sexual maturation and pubertal growth.

Around 30 cases of AEXS have been reported so far, the majority of which are male patients (1, 3, 5–12). There is a wide phenotypic variability among patients, which may be due to their genetic finding, including microscopic tandem duplications, microscopic deletions and inversions at 15q21.2 (1–3, 11, 13, 14). The most characteristic clinical feature is bilateral gynecomastia, which typically appears before or during the onset of puberty, mainly due to local conversion of circulating androgens from the adrenal grand (adrenarche) into estrogens (3, 14). The degree of gynecomastia may vary, from mild to severe; however, most of the reported patients underwent a mastectomy at an early age (3, 14). Other clinical features may include accelerated bone age and short adult height due to early fusion of epiphyses (3, 6, 10). In addition, some male patients with AEXS may present with mild follicle stimulation hormone (FSH) dominant hypogonadotropic hypogonadism during puberty, which may inhibit normal testicular growth and virilization (1, 3, 13, 14). Hypogonadotropic hypogonadism may remain in adulthood, however, fertility has been reported to remain unaffected (1, 3, 13, 14). As for female patients, only a few cases have been reported so far, probably due to their milder phenotype or lack of any striking symptoms that would lead these patients to a physician for further investigation (3, 13). In female patients with AEXS, estrogen excess, which is less significant than in males, may lead to premature thelarche, accelerated bone age, short adult height, macromastia, enlarged uterus and/or menstrual irregularities (3, 6, 10, 14). A helpful tool for the clinical diagnosis of AEXS in boys has been previously proposed and includes the following criteria: bilateral and Tanner stage>2 gynecomastia, onset of gynecomastia after the age of 5 years and before the age of 14 years, exclusion of other well-known causes of gynecomastia and having a genetic trait (autosomal dominant) (3). Interestingly, despite the estrogen excess, serum estradiol may be normal in 20% of the patients (3, 12) and some authors suggest using the estradiol/testosterone ratio as index of aromatization (12). A definite diagnosis, though, should be confirmed through genetic testing (3).

In order to decrease the circulating levels of estrogens and, therefore, their action on different tissues, aromatase inhibitors (AIs) are suggested for use in male patients with AEXS (4). AIs were initially developed for the palliative or adjuvant treatment of estrogen-dependent breast cancer as they act by inhibiting the intracellular conversion of androgens to estrogens (4, 15). Up to now, AIs are not approved for any pediatric indication, but are used off-label in children with a few, very rare disorders, as their efficacy is well established. These include AEXS, Peutz-Jeghers syndrome, McCune-Albright syndrome, functional follicular ovarian cysts and testotoxicosis (4, 15, 16). Interestingly, AIs have been tested in boys with idiopathic short stature in order to promote growth, however there are no convincing data to support the beneficial effect of AI therapy on adult height in these boys so far (17). In boys with pubertal gynecomastia, the use of AIs was not found to be effective and is, therefore, not recommended (18). Nowadays the most commonly used AIs in children are the third generation AIs, letrozole and anastrozole. Up to now, there are no pharmacological studies regarding the optimal dose of AIs in children, so children are usually being treated with the same dosage of AIs as adults (15). In male patients with AEXS, AIs are found to increase testosterone levels, promote virilization, increase testicular volume and control the development of gynecomastia (3, 6). A recent study from Binder et al. examined for the first time the effects of a long-term treatment with anastrozole on growth on four male patients with AEXS and showed that AIs may promote adult height when started early (5). There are no reports regarding the use of AIs in females with AEXS so far. In

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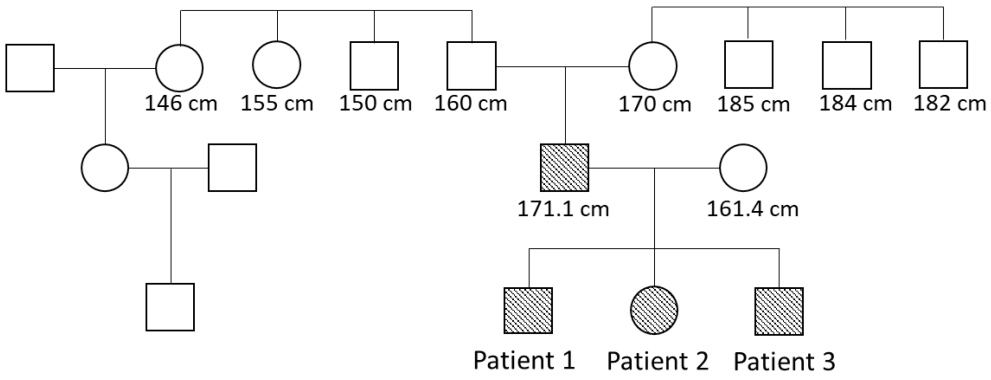

FIGURE 1
Pedigree. Patients with heterozygous microdeletion in *CYP19A1* are indicated by filled forms. The numbers are adult heights.

this study, we present the phenotypic characteristics of a family with four members with AEXS, and additionally show the long-term follow-up, from childhood to adulthood, of three AIs-treated patients (two male, one female).

Materials and methods

Clinical data

We present a family of German origin in which early puberty and short adult height has been reported over three generations in males and females (Figure 1). The patients have been reported in a previous study (2), which investigated the genetic basis of AEXS in 6 unrelated patients. The index patient, his parents and his two siblings were examined and treated at the Division of Pediatric Endocrinology and Diabetes, Department of Pediatrics and Adolescent Medicine, University Medical Center in Ulm, Germany. Data from the patients were collected and analyzed retrospectively. In all three children the pubertal stage (development of breast, genitalia and pubic hair) was documented according to the classification by Tanner. Testicular volume was measured using an orchidometer according to Prader (19). Target height was calculated according to Tanner et al. (20). For this study, target height included the height of the affected parent (uncorrected). Height-SDS was calculated using the least mean squares method based on German references (21). Bone age and prognosis of adult height were estimated using the Greulich and Pyle atlas (22) and the Bayley and Pinneau tables for German children (23), respectively.

DNA sequencing and laboratory data

Leukocyte genomic DNA samples were obtained from the parents and siblings of the index patient. Genomic abnormalities involving *CYP19A1* exons and/or its flanking regions were examined by comparative genomic hybridization (CGH) using a custom-made oligoarray or a catalog human array, as described previously (2).

During treatment with aromatase inhibitor letrozole, assessment of liver function, lipids, hemoglobin and hematocrit were performed every 6-12 months. Hormonal analysis was performed at each appointment and included testosterone, androstenedione, estradiol, DHEA sulfate (DHEAS), luteinizing hormone (LH) and FSH. Hormone serum concentrations were determined with electrochemiluminescent assay (ECLIA). Testosterone was measured by Elecsys Testosterone assay on Cobas pro analyzer (e 801 module) (Roche Diagnostics, Switzerland). The measuring range for testosterone was 0.087 −52.0 nmol/L (defined by the Limit of Detection and the maximum of the master curve). Estradiol was measured by Elecsys Estradiol assay on Cobas pro analyzer (e 801 module, measuring range: 18.4−11010 pmol/L) (Roche Diagnostics, Switzerland). Androstenedione was measured by Elecsys Androstenedione assay on Cobas pro analyzer (e 801 module, measuring range: 0.525−34.9 nmol/L) (Roche Diagnostics, Switzerland). LH was measured by Elecsys LH assay on Cobas pro analyzer (e 801 module) (Roche Diagnostics, Switzerland). FSH was measured by Elecsys FSH assay on Cobas pro analyzer (e 801 module) (Roche Diagnostics, Switzerland).

In vitro studies

For the *in vitro* studies, adipose stromal cells (ASC) were isolated from an adipose tissue sample of the index patient during elective surgery (24). Control ASCs were isolated from subcutaneous adipose tissue obtained from reduction surgery. The cells were grown and aromatase activity was measured by the tritium water release assay in 24-well plates using [1β-^{3}H] androstenedione (PerkinElmer, Rodgau, Germany) as substrate, as described previously (25, 26). Briefly, incubations with test substances were done for 24 h in cells preconditioned with serum-free medium. Aromatase activities were normalized to the protein content (27). Peripheral blood leukocytes (PBL) were isolated from the index patient and a healthy control using standard procedures. Two million PBL in RPMI1640 medium with fetal calf serum (FCS) were inoculated per well and were

directly treated with cortisol or vehicle (ethanol) for 24 h. Values from PBL were normalized to cell number. All conditions were tested in quadruplicate.

From aliquots of ASCs and PBL, total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) with DNAse digestion. Therefrom, cDNA was synthesized with the High-Capacity cDNA Archive Kit (Applied Biosystems, Darmstadt, Germany) using random hexameric primers. Specific assays based on the Universal Probe Library system (Roche, Mannheim, Germany) were established according to the manufacturer's standard instructions, as described previously (27). The following assays were used (name, forward primer, reverse primer, probe): full length (exons IX–X), CAA ACC CAA TGA ATT TAC TCT TGA, ACC ATG GCG ATG TAC TTT CC, probe 76; promoter I.1, GTG CTC GGG ATC TTC CAG, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.8, TTG GAC CCC AGA CTT AAG GA, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.4, CAG CCC ATC AAA CCA GGA, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.5, CAG GAT TGA GCA CAC AGG AC, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.7, AGG GGT GAA ATC AGC AAG G, CAT GGC TTC AGG CAC GAT, probe 9; promoter I-f, GAC CAG CAG ACC CAG GAC, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.2, GCT GAT CCC AGT TCT GAA GAG, TCA GAG GGG GCA ATT TAG AG, probe 30; promoter I.6, CAG GAT GTT AGC TGC TCT TCG, CAT GGC TTC AGG CAC GAT, probe 9; promoter I.3, CTT GCC TAA ATG TCT GAT CAC ATT A, CAT GGC TTC AGG CAC GAT, probe 9; promoter II, CCC TTT GAT TTC CAC AGG AC, CAT GGC TTC AGG CAC GAT, probe 9; and GAPDH, AGC CAC ATC GCT CAG ACA C, GCC CAA TAC GAC CAA ATC C, probe 60. All samples were analyzed in duplicate. Relative gene expression was normalized to GAPDH mRNA levels using the comparative cycle threshold method (27).

Statistical evaluation of the *in vitro* experiments was done with 2-sided Student's t-test (with Bonferroni correction as necessary) as all data sets were normally distributed. Graphs with height trajectories were designed using Graph Pad Prism 7 (Graph Pad Software Inc., San Diego, CA, USA). Percentiles for height were drawn using German reference data (21). Written informed consent was obtained from the patients. The study was approved by the ethics committee of the University of Ulm (247/18) and complies with the declaration of Helsinki.

TABLE 1 Clinical characteristics at presentation of patients 1, 2 and 3 with aromatase excess syndrome.

	Patient 1	Patient 2	Patient 3
Sex	Male	female	male
Age (years)	8	6	3
Height (cm) [SDS]	139.9 [+1.7]	126.3 [+1.7]	100.0 [+0.9]
Gynecomastia (Tanner stage)	B2	B3	no
Testes volume (ml)	1	–	1
Pubic hair (Tanner stage)	PH1	PH1	PH1
Bone age (years)	13.5	10.5	5.5
Height prediction (cm) [SDS]	159.8 [-3.1]	146.7 [-3.4]	167 [-2.0]
Target height (cm)* [range: ± 2SD]	172.8 [164.3-181.3]	159.8 [151.3-168.3]	172.8 [164.3-181.3]

SD, standard deviation; SDS, standard deviation scores.
*Target height was calculated according to Tanner and therefore includes the height of the affected parent.

Results

Patient 1 (index patient)

The index patient was primarily seen in our outpatient clinic at the age of 8 years due to bilateral gynecomastia (Tanner stage B2) and accelerated growth (Table 1). Physical examination revealed normal prepubertal external genitalia. His bone age was advanced by 5.5 years and the predicted adult height according to the bone age was 159.8 cm, which was significantly lower than his estimated target height (Table 1). In the laboratory examination, gonadotropin and testosterone levels were prepubertal and estradiol was undetectable. Hyperthyroidism, hyperprolactinemia, liver disease and renal failure were excluded. Tumors, including testicular tumors (germ cell, Sertoli or Leydig cell tumor) and extragonadal tumors, were excluded by testicular ultrasound and normal hCG, β-hCG and AFP levels in laboratory exams. Urinary steroid excretion analysis revealed normal findings. The patient's karyotype was 46, XY. Regarding his family history, his father mentioned an early, accelerated growth with a relatively short adult height. The paternal grandfather, two grandaunts and one granduncle reached an adult height of between 150 and 160 cm (Figure 1). There was no reported gynecomastia in the family, as well as no infertility problems.

The index patient was followed regularly in our department for progression of growth, puberty and gynecomastia. Already at the age of 9 $^{10/12}$ gynecomastia progressed to Tanner stage B4 and due to psychosocial stress, a bilateral mastectomy was performed as a permanent solution. Start of puberty with increase of testicular volume (4 ml) occurred at the age of 11 $^{1/12}$ years. With start of puberty, gynecomastia progressed again, and in the laboratory examinations serum estradiol levels increased above normal range, whereas testosterone and androstenedione levels remained normal or low (Figure 2). At the age of 13 $^{2/12}$ years a second bilateral mastectomy was performed (Tanner stage B3). Since pubic hair development progressed, there was a slow development of testicular volume, reaching the maximum of 8 ml at the age of 18 years. Regarding his growth pattern, the patient had an early, accelerated growth with growth arrest at age 14 years (adult height: 168 cm, -1.8 SDS) (Figure 3).

Presuming an aromatase excess syndrome and while the first genetic findings causing AEXS were published in the literature at that time (1, 10), a genetic analysis of the aromatase gene was performed, which revealed an approximately 0.3-Mb heterozygous deletion in the upstream region of *CYP19A1*. This microdeletion included 7 of the 11 non-coding exons 1 of *CYP19A1*, all exons of *GLDN*, and exons 2-43 of *DMXL2* (2). This deletion is predicted to cause abnormal splicing between *DMXL2* exon 1 and *CYP19A1* coding exons (Figure 4). Since DMXL2 is a widely expressed gene

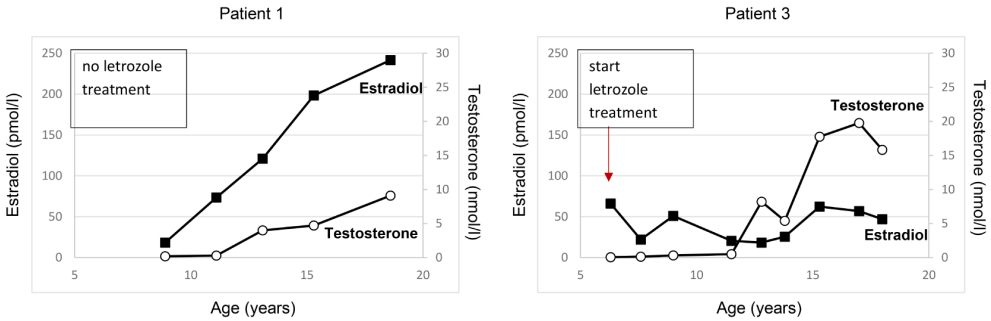

FIGURE 2
Estradiol and testosterone serum concentrations over time in the two male patients with AEXS. Patient 1 received no aromatase inhibitor treatment till age 19 years. Patient 3 was started on letrozole treatment at the age of 6 years and continued till the age of 18 years. Testosterone levels are shown in white circles, estradiol levels are shown in black squares.

(GTEx Portal, https://gtexportal.org/home/), this abnormal splicing likely leads to overexpression of *CYP19A1*. Genetic analysis was consecutively performed to his parents and two siblings; his father, brother and sister were all found to carry the same microdeletion as the index patient (2) (Figure 4).

A therapy with letrozole 2.5 mg daily was initiated at the age of 19 years in order to increase testosterone levels and promote testicular volume (7). Testosterone enanthate injections were added to the treatment after 1 year of letrozole therapy because of low testosterone levels and the wish of the patient, due to subnormal masculinization and sparse facial and body hair. Letrozole was stopped and monotherapy with testosterone was continued for 1 year, but there was no change regarding testicular volume and testosterone levels. From the age of 21 years on, the patient was on monotherapy with letrozole, starting again with a full dose of 2.5 mg daily. During treatment testosterone, androstenedione, LH and FSH levels increased gradually, while estradiol levels decreased to normal (Table 2). Supraphysiological concentrations of testosterone up to 33.3 nmol/l (reference range: 8.60-29.00 nmol/l) were measured during letrozole treatment. In this case, letrozole dose was gradually

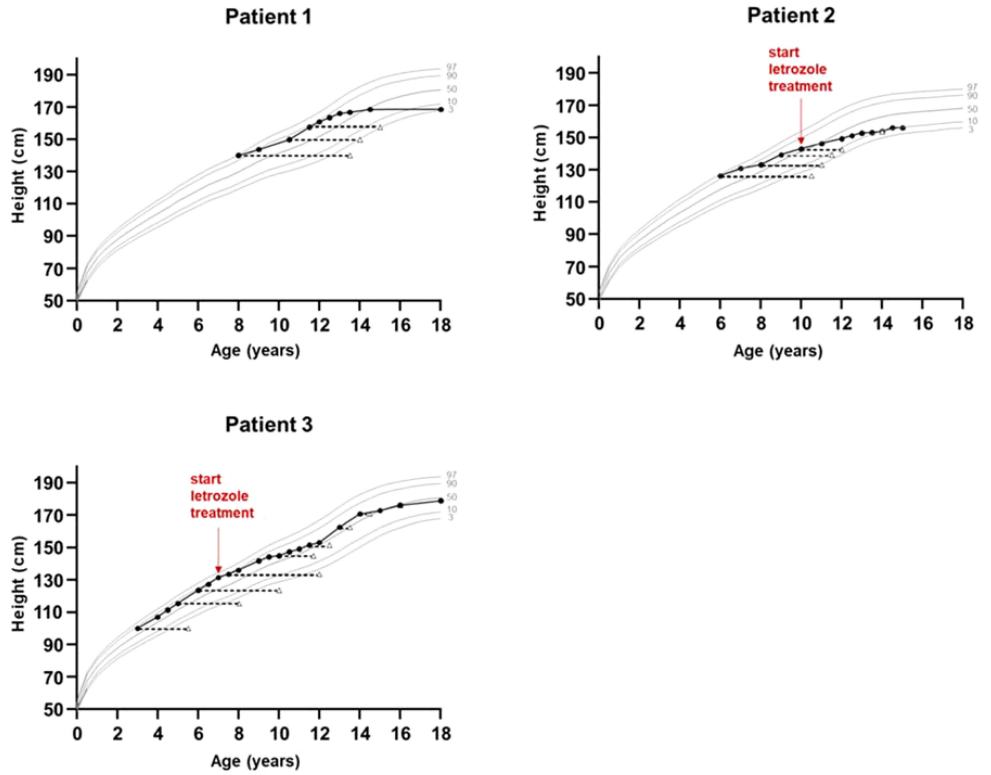

FIGURE 3
Growth charts of patients with aromatase excess syndrome. Bone age is shown in triangles. Shown are the 3rd, 10th, 50th, 90th and 97th height percentiles according to German reference data for boys (patients 1 and 3) and girls (patient 2).

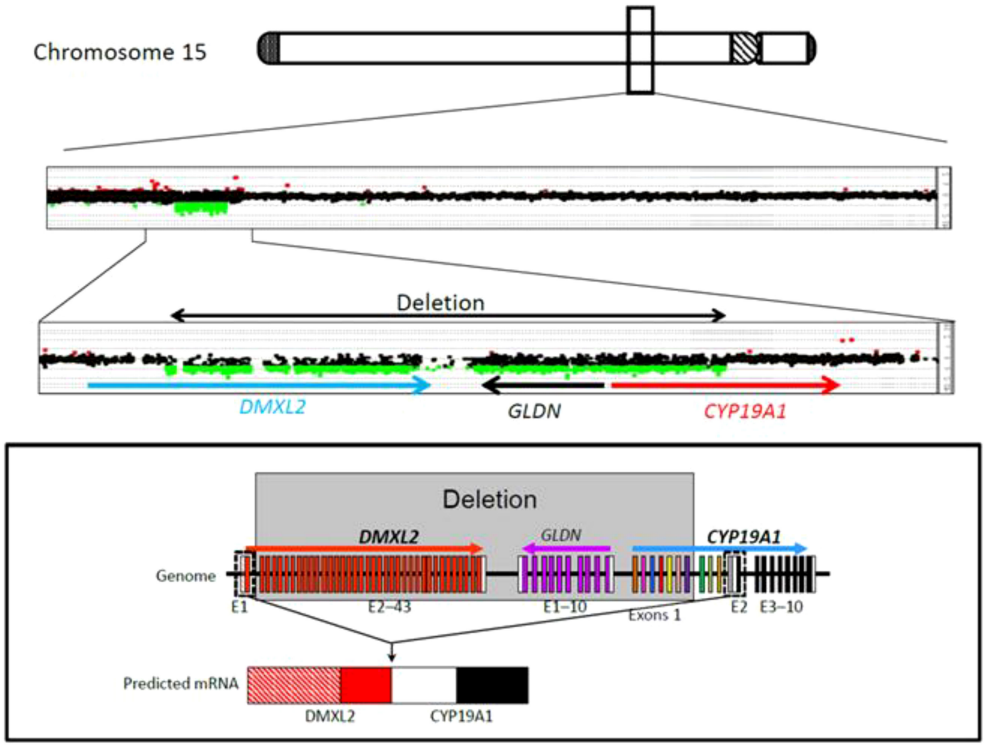

FIGURE 4
CGH analysis of the four family members with aromatase excess syndrome, revealing a heterozygous microdeletion in the upstream region of CYP19A1.

reduced and testosterone levels were measured again. When the dose was decreased to 0.1 mg per day, treatment was terminated and the patient was reevaluated after 6 months; interestingly, testosterone levels were found to be low and estradiol levels were elevated, so that treatment with low-dose letrozole was restarted. Letrozole dose was titrated down to a minimum of 0.015 mg/day at the age of 25 years which is still continued (Table 2). Low-dose letrozole treatment resulted in gradual promotion of testicular volume (adult volume was achieved at the age of 24 years) and improvement of physical strength and libido. The patient and his wife have one child. No gynecomastia was observed under treatment. Vertebral abnormalities, as assessed by spinal X-rays, were not observed and annual controls of markers of calcium metabolism revealed normal findings. Semen evaluation was refused.

Patient 2 (sister of index patient)

The sister of the index patient presented at the age of 6 years with premature thelarche (Tanner stage B3), accelerated growth and advanced bone age of 10 $^{6/12}$ years (Table 1). At this time point the adult height prognosis was estimated to be 146.7 cm (range ±2SD: 140.9-152.5 cm) (23), which was significantly lower than her estimated target height (159.8 cm, range ±2SD: 151.3-168.3 cm). Laboratory examinations revealed low gonadotropin levels and a serum estradiol level of 77 pmol/l. A gonadotropin-releasing hormone stimulation test excluded central precocious puberty, and an off-label therapy with tamoxifen, an estrogen receptor antagonist, was initiated at the age of 7 years due to precocious thelarche and growth acceleration. At the age of almost 10 years and because of vaginal bleeding, the patient was started on a GnRH analog. At the age of 11 years and after receiving the genetic diagnosis of AEXS, 2.5 mg letrozole daily were added to the therapy. At that time her height was 146.3 cm and her bone age was 12 years (Figure 3). At the age of 12 years treatment with both drugs was stopped and menarche occurred at age 13 $^{6/12}$ years. The patient reached an adult height of 158 cm (-1.6 SDS) (21), which was within her estimated target height range (mean target height: 159.8 cm, range ±2SD: 151.3-168.3). Pelvic ultrasound examinations revealed no pathological findings, and following the next years no menstrual irregularities were reported till the age of 16 years, when the use of a birth control pill was initiated due to her wish for contraception. All markers of calcium metabolism are up to present within normal range.

Patient 3 (brother of index patient)

A younger brother of the index patient presented at the age of 2 $^{11/12}$ years with accelerated growth and bone age (Tanner stage B1, PH1, bone age 5 $^{6/12}$ years) (Table 1). Following the diagnosis of his brother, a genetic analysis was performed and confirmed AEXS. In cognition of his brother's history, treatment with 1.25 mg letrozole

TABLE 2 Laboratory findings before and during treatment with aromatase inhibitor in male patients 1 and 3.

Patient 1					
Age (years)	18	22	25	29	35
Daily aromatase inhibitor dosis	–	2.5 mg	0.02 mg	0.015 mg	0.015 mg
Testosterone (nmol/l)	9.11 (9.70-27.73)	29.82 (9.70-27.73)	17.51 (8.63-29.00)	17.58 (8.63-29.00)	26.41 (8.63-29.00)
Androstenedione (nmol/l)	7.63 (4.50-15.00)	13.23 (2.44-12.57)	5.52 (2.44-12.57)	3.07 (2.44-12.57)	3.11 (0.98-5.31)
Estradiol (pmol/l)	241.6 (27.90-156.40)	23.50 (27.90-156.40)	70.49 (27.90-156.40)	83.71 (27.90-156.40)	158.60 (27.90-156.40)
LH (mIU/ml)	2.07	44.80	4.50	N/A	N/A
FSH (mIU/ml)	1.20	24.90	3.00	N/A	N/A
Patient 3					
Age (years)	6	8	15	18	28
Daily aromatase inhibitor dose	–	2.5 mg	0.6mg	0.3 mg	–
Testosterone (nmol/l)	<0.09 (0.13-1.01)	0.14 (5.89-29.47)	17.75 (9.70-27.73)	15.81 (9.70-27.73)	4.47 (8.63-29.00)
Androstenedione (nmol/l)	N/A	N/A	9.36 (2.44-12.57)	8.66 (4.50-15.00)	1.60 (0.98-5.31)
Estradiol (pmol/l)	66.08 (<73.40)	22.00 (<73.40)	62.40 (27.90-156.40)	47.00 (27.90-156.40)	175.50 (27.90-156.40)
LH (mIU/ml)	<0.10	0.40	2.90	2.18	6.65
FSH (mIU/ml)	0.67	2.21	5.95	6.71	3.94

LH, luteinizing hormone; FSH, follicle stimulating hormone.

daily was started at the age of 6 $^{4/12}$ years, in order to control growth, sexual maturation and the development of gynecomastia. At that time his height was 127.3 cm and his bone age was 10 years (Figure 3). The dose of letrozole was soon increased to 2.5 mg per day; serum levels of gonadotropins, testosterone and estradiol were regularly screened during treatment (Table 2). Letrozole treatment during puberty resulted in hormonal balance between estradiol and testosterone (Figure 2). Puberty started at the age of 10 years and testicular volume developed normally. Due to elevated testosterone levels from the age of 12.5 years, the dose of letrozole was gradually decreased reaching the minimum of 0.3 mg per day at age 16 years. He continued with this dose till the age of 18 years. Due to the patient's wish a bilateral, prophylactic mastectomy was performed and treatment with letrozole was stopped thereafter. The patient reached an adult height of 178.8 cm (target height: 172.8 cm, range ±2SD: 164.3-181.3 cm). Virilization and adult testicular volume were reached at age 16 years. Gynecomastia was not observed. We did not observe any side effects during treatment with letrozole. Markers of calcium metabolism in blood remained within normal range. Semen evaluation was refused.

Patient 4 (father of index patient)

The index patient´s father had an early, accelerated growth with a relatively short adult height (Figure 1), which was, however, within his estimated target height. He mentioned a delayed puberty and his first shave was at the age of 17 years. During his whole life he mentioned sparse body and facial hair. He had no gynecomastia. Laboratory examination revealed elevated estradiol and low testosterone levels (data not shown).

Results from *in vitro* studies

To further verify manifestations of aromatase excess resulting from the microdeletion found via CGH, we analyzed aromatase activity and mRNA-expression in ASCs, which normally express significant amounts of aromatase, and in PBL, which normally show only marginal signs of aromatase expression. The pattern of aromatase activities resulting from various (combinations of) inducers in ASCs from the index patient appeared almost normal, as compared with a group of 12 controls matched for equal duration of cultivation of the ASCs (Figure 5A). It revealed the typical induction by cortisol in the presence of FCS, which in the absence of serum can be replaced by a growth-factor like platelet-derived growth factor (PDGF), and which activates promoter I.4. In normal ASCs, the activities induced thereby are roughly equal (28). However, in the patient´s cells the induction in the presence of FCS was significantly stronger, suggesting some additional induction mediated by another pathway. Analysis of the appearance of the typical alternative, untranslated first exons in the aromatase mRNA

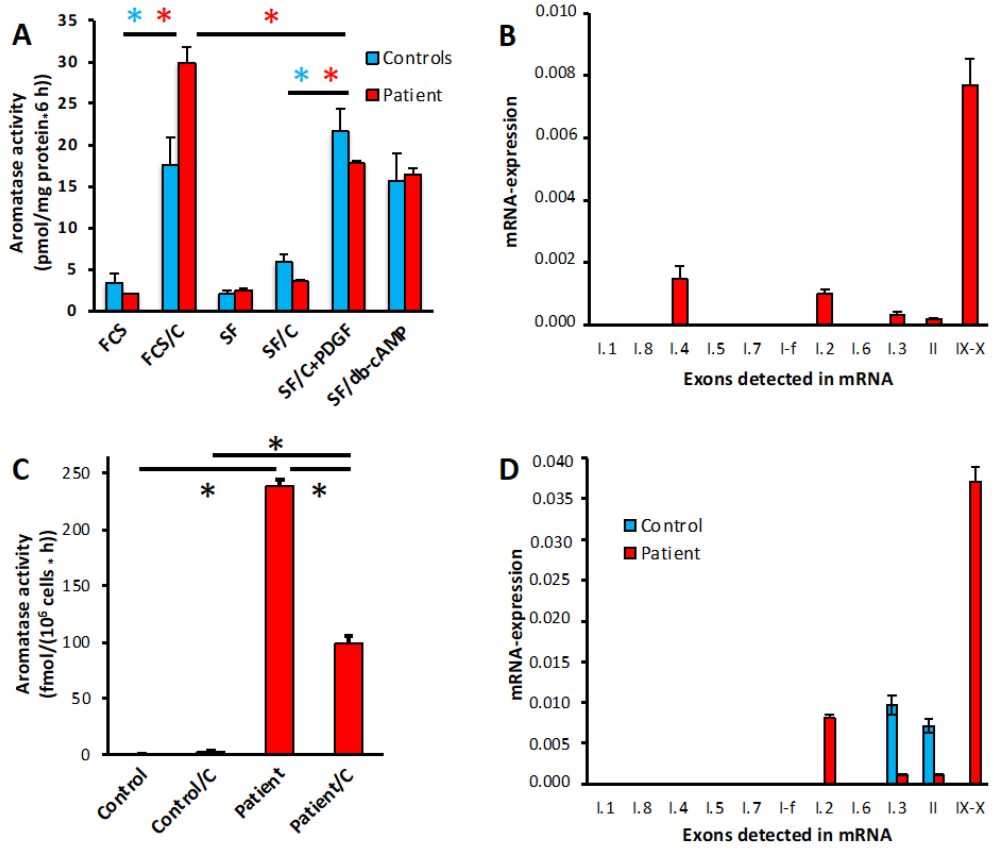

FIGURE 5
Aromatase activity and promoter usage in ASCs and PBL. **(A)** Aromatase activity in patients ASCs treated in the presence (FCS) or absence (SF) of serum with vehicles or the indicated inducers: 1 μM cortisol **(C)**, 0.5 nM PDGF-BB (PDGF) or 1 mM dibutyryl-cAMP (db-cAMP). Normal control data were taken from a matched group of donors (n=12) undergoing elective adipose tissue reduction surgery, which were cultured under the same conditions as the patient's cells. **(B)** Promoter usage in patient ASCs. Promoter specific mRNAs are detected by qPCR and are indicated in their normal order on the DNA, IX-X indicates full-length transcripts. Expression is normalized to *GAPDH*. **(C)** Aromatase activity in PBL from a unaffected donor (control) or the patient, treated with vehicle (ethanol) or 1 μM cortisol **(C)** for 24 h. **(D)** Promoter usage in PBL from the same donors as used in **(C)**. Aromatase activities were assayed in quadruplicate replicates, qPCR was done in duplicates. *, p<0.001.

allows determination of the promoters used for induction. It revealed the appearance of exon I.2-containing mRNAs, in addition to mRNAs containing exons I.4, I.3 and II in normal ASCs (Figure 5B), thus confirming the CGH-results.

Aromatase activity (Figure 5C) and expression of full-length mRNA containing exons IX and X (Figure 5D) are barely detectable in normal PBL (where appearance of exons I.3 and II indicates the expression of some truncated transcripts). In contrast, the patient's PBL exhibited significant aromatase activity and full-length mRNA, driven mostly by exon I.2 containing transcripts originating from the allele with the microdeletion. Interestingly, in these PBL cortisol decreased aromatase activity, which might be attributed to cortisol acting on the *DMXL2* promoter.

Discussion/conclusion

AEXS is a rare disorder characterized by enhanced extraglandular aromatization of androgens and estrogen excess (1). In order to increase adult height, promote testicular development and prevent gynecomastia, 3rd generation AIs are used off-label in male patients with AEXS (3, 5). Here, we presented a family with four members with AEXS, in which the three offspring were treated with the 3rd generation AI, letrozole. The 8 year old index patient, who presented with gynecomastia, tall stature and accelerated bone age, was started on letrozole treatment at the age of 19 years, which resulted in sexual maturation. The brother of the index patient presented at the age of 3 years with accelerated growth and bone age. Letrozole treatment was started at the age of 6 years and resulted in achieving an adult height within his estimated target height range, prevention from the development of gynecomastia, and promotion of sexual maturation. The sister of the index patient, who had a history of premature thelarche, accelerated growth and bone age, was treated at first with an estrogen receptor antagonist followed by a treatment with letrozole and GnRH analog and resulted in reaching an adult height within her estimated target height range.

Prepubertal gynecomastia is not a common finding in young boys. AEXS is a rare cause of prepubertal, bilateral gynecomastia in boys, occurring during adrenarche in most of the reported cases with AEXS (3). The differential diagnosis of gynecomastia mainly includes secondary causes like chromosomal anomalies (Klinefelter

syndrome), enzyme deficiencies (e.g. 17β-hydroxysteroid dehydrogenase deficiency, 21-hydroxysteroid dehydrogenase deficiency, 17α-hydroxylase deficiency syndrome deficiency etc.), liver cirrhosis, tumors (e.g. hCG-producing tumors, choriocarcinoma, germ cell tumors, estrogen-producing tumors etc.), renal disease and use of specific drugs (e.g. aldosterone receptor blockers, anti-hypertensive drugs, psychotropic drugs etc.) (3). Physiological pubertal gynecomastia is a common condition that usually occurs around the age of 14 years in otherwise healthy boys, has a slow progression and resolves spontaneously within 24 months (3, 18). Accelerated growth, advanced bone age, short adult height, underdeveloped testes during puberty and sparse body and facial hair are suggestive of estrogen excess and should provoke further laboratory and genetic testing. As in our case, a positive family history including cases of gynecomastia and short adult height, is definitely a strong indicator for further investigation.

Gynecomastia in young male individuals may have a significant impact on their self-esteem and quality of life. The majority of reported male cases has undergone mastectomy as early as the age of 12 years (3, 5). Although rare, there are cases of recurrence of gynecomastia after mastectomy in patients with AEXS (5). In the present study, prophylactic and early letrozole use prevented the appearance of gynecomastia during puberty in one patient. Binder et al. showed, similarly, that gynecomastia regressed within a few months after start of treatment with a third generation non-steroidal AI, anastrozole, in 4 boys with AEXS (5). Regarding recurrence of gynecomastia, the authors suggested timely mammoplasty after cessation of treatment with anastrozole (5). In our study, the brother of the index patient underwent a prophylactic mastectomy after cessation of AI treatment; the patient has not developed gynecomastia already ten years after end of treatment with letrozole. A close follow-up is suggested, however, in these patients. Patients with an early diagnosis of AEXS, preferably before onset of puberty, may benefit from a prophylactic treatment with AIs, not only regarding gynecomastia, but also regarding growth, as explained below.

Apart from the positive effects on the development of gynecomastia, the use of AIs in male patients with AEXS has been found to improve adult height, promote virilization and increase testicular volume (5, 7, 10, 12, 29). So far, anastrozole has been used in the majority of studies with patients with AEXS, while letrozole has been used in combination with growth hormone treatment in only one study (12). Both anastrozole and letrozole suppress estrogen production by 97-99% and are highly selective (4). In the current report, letrozole was selected due to its higher potency in inhibiting aromatase activity. Letrozole is, like anastrozole, rapidly absorbed, but has a longer half-life of 2 to 4 days, leading to higher plasma testosterone concentrations (4, 30). Indeed, supraphysiological levels of testosterone were observed during letrozole treatment in the two male patients in the present study. Because of increased testosterone levels, the dose of letrozole has been titrated down to a minimum of 0.015 mg per day in the index patient and 0.3 mg per day in the brother of the index patient. As of note, the index patient was started on letrozole in adulthood, while his brother in childhood. The increase in testosterone levels was accompanied by a concomitant rise in LH and FSH concentrations; clinically an increase in testicular volume was observed. Our findings are supported by previous research showing also a supraphysiological rise in testosterone concentrations and rapid testicular growth due to increase in gonadotropin secretion in boys with idiopathic short stature treated with letrozole (30). Suppression of estrogen biosynthesis by letrozole decreases the negative feedback control of gonadotropin secretion and raises concentrations of serum FSH and LH (30). Regular follow-up of these hormones is suggested and appropriate adjustments of letrozole dose should be considered during letrozole treatment.

Another significant effect of early administration of AIs in patients with AEXS is on adult height (5). Estrogen excess already before the onset of puberty accelerates skeletal maturation and growth and causes premature closure of the epiphyses (3, 5). Adult heights of untreated patients with AEXS are found to range between -2.5 and 0 standard deviations of normal (3). So far, there is only one study that has investigated the long-term effect of AIs (anastrozole) on adult height in patients with AEXS (5). This study showed that early initiation of 1.0 mg anastrozole therapy resulted in improved adult height in two male patients, compared with the initial prediction (+6.9 and +8.1 cm) (5). The results of our current study are in agreement with previous findings, as the younger brother, who was started early on AI treatment reached his estimated target height and improved his initial prediction (+8.4 cm). Of note, this is the youngest reported male patient with AEXS treated with AIs, compared to previous studies (5). Adult height of patients with AEXS should be carefully assessed in terms of uncorrected target height, especially if one of the parents is affected with AEXS, as in the present study. While the two patients in this study reached their genetic potential based on midparental height, midparental height may underrepresent true target height in case the father's height was compromised due to AEXS. Still, early intervention, when possible before puberty onset, should be considered in patients with AEXS in order to improve adult height.

The female patient in the present study reached an adult height within her estimated target height range. However, her course was complicated by the presence of precocious puberty, as well as by the administration of an estrogen receptor antagonist and a GnRH analog before AI treatment. Therefore, although her height prognosis was improved, a clear conclusion about the effect of letrozole use on height development cannot be drawn. Regarding females with AEXS, the main challenge remains the diagnosis, especially when it comes to sporadic cases, when there are no affected male members in the same family. In the literature there are only a few reports on female patients with AEXS describing their phenotype, which includes premature thelarche, accelerated bone age, short adult height, macromastia, enlarged uterus and/or menstrual irregularities (3, 6, 10, 14). There is no available information regarding AI treatment, mainly due to concerns that

treatment with AIs may cause ovarian overstimulation in females (31). Since a positive result of AI treatment on adult height has been proved in males, a short-term AI use should be considered in females with AEXS in order to improve adult height.

In AEXS, phenotypic severity seems to be determined by the expression levels of *CYP19A1* due to the type of genomic rearrangement (1–3, 11, 13, 14). As such, patients with inversions are found to show an early disease onset with severe gynecomastia, advanced bone age and short adult height, while patients with duplications show mild gynecomastia with pubertal onset and normal adult height. Patients with deletions are found to exhibit an intermediate phenotype (2, 3, 13). Interestingly, in the present study we observed a phenotypic variety within family members carrying the same microdeletion of *CYP19A1*. This finding needs to be confirmed also in other family studies in order to identify the factors responsible for this phenotypic variety.

Our study also verified atypical expression patterns of *CYP19A1*/aromatase in affected patients. Analysis of aromatase activity and mRNA-expression in ASCs, which normally express significant amounts of aromatase, revealed a potentiation of inducible activity in the presence of the mutated allele when cortisol and FCS were used for induction. However, due to the young age of the index patient at the time of surgery it was not possible to obtain significant numbers of age matched controls. Therefore, matching was based on equal duration of cultivation of the ASCs, as there is a significant influence of duration of *in vitro* cultivation on aromatase activity of ASCs (32). Despite this limitation of the experiments with ASCs, it is obvious that the mutated allele is linked to an altered pattern of aromatase activities in these cells. Furthermore, and perhaps more important, we showed that the mutated allele is sufficient for induction of functional aromatase expression in cells not normally doing so. Our results further indicate that also in cells with ectopic expression of aromatase its activity may be regulated by hormonal factors like cortisol, which obviously does not always act as an inducer of aromatase in these patients' cells/tissues. This may at least in part explain the large phenotypic variety seen in AEXS patients.

In most of the previous reports, AI use was terminated when adult height was reached (5). A long-term use of AIs extending to adult life in patients with AEXS has been previously suggested (3), however studies are lacking. In the present study we examined the use of low-dose letrozole during adult age in one patient. During adult age, AI use at very low doses resulted in normal serum levels of estrogens and androgens. Clinically, AI treatment improved physical strength and libido. A long-term use of AIs at a low dose should be considered in adult male patients with AEXS. It is important, though, that more cases are documented. The consequences of long-term hyperestrogenemia in patients with AEXS regarding coronary heart disease as well as breast and prostate cancer are unknown, so a regular follow-up for these conditions is suggested.

Markers of bone health (calcium metabolism) in our two long-treated patients showed no pathological findings. In addition, no history of bone fractures was reported. Patients with AEXS are expected to have normal or even increased bone mineral density (5, 7). During treatment with AIs, bone health is not expected to be impaired as estrogen levels are regularly screened and are aimed to remain at normal levels. Accordingly, Binder at al. showed that treatment with anastrozole did not reduce bone density as controlled by DEXA scan in two patients (5). No detrimental effects of AI use on bone health have been reported in males so far (3).

Limitations of the present study is the limited number of included patients and the absence of a control group. However, AEXS is an extremely rare disorder with only a few reported cases so far, therefore our study adds new knowledge to the current literature.

In conclusion, the present study provided information on the clinical phenotypes of four family members with AEXS as well as atypical expression patterns in ASCs and PBL. Long-term low dose letrozole treatment in adulthood promoted testicular growth and improved physical strength in one adult patient. In the second male patient, initiation of letrozole treatment before onset of puberty resulted in improved adult height and prevented from the appearance of gynecomastia. The female patient, who had a history of precocious puberty, received a combined treatment including letrozole, which resulted in reaching her estimated target height. While AEXS is a rare disorder, diagnosis should be suspected in cases of prepubertal gynecomastia, accelerated growth and advanced bone age, and confirmed through genetic testing. More reports with long-term follow-up data are still needed in order to prove the positive effects of AI treatment on adult height and gynecomastia.

Data availability statement

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

Ethics statement

The studies involving humans were approved by University of Ulm, 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. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

EG: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. SB: Writing – review & editing, Data curation. SZ: Writing – review & editing, Data curation. CD: Writing – review & editing. JV: Writing – review & editing. MF: Writing – review & editing, Methodology. AK: Writing – review & editing, Methodology, Data

curation. MS: Writing – review & editing, Validation, Methodology, Investigation, Data curation. MW: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization.

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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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Serum exocrine pancreas enzymes are biomarkers of immunotherapy response in new-onset type 1 diabetes

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Introduction: The immune-mediated destruction of insulin-producing β-cells characterizes type 1 diabetes. Nevertheless, exocrine pancreatic enzymes, including amylase, lipase, and trypsin, are also significantly reduced in type 1 diabetes. With an immunotherapy now approved to treat early-stage type 1 diabetes, biomarkers to delineate response to treatment are needed. No study has yet evaluated whether serum exocrine pancreatic enzymes could delineate immunotherapy responders and non-responders.

Methods: In this novel study, we sought to identify longitudinal trends in the most commonly measured circulating exocrine enzymes before and after treatment with anti-thymocyte globulin (ATG) and pegylated granulocyte colony-stimulating factor (GCSF) in individuals with new-onset type 1 diabetes (n=34). We defined response to immunotherapy as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at two years post-treatment. In the overall study (n=89), 42% of treated and 17% of placebo participants met this definition. Due to constraints of sample availability, we compared longitudinal serum amylase, lipase, and trypsin levels in a subset of responders to therapy (n=4-6), placebo "responders" (n=2), treated non-responders (n=16), and placebo non-responders (n=10).

Results: There were no differences in amylase levels between groups at baseline or six months post-treatment. Baseline levels of lipase and trypsin tended to be lower in responders; however, these variations were not significant in this small study sample. Lipase and trypsin improved to 115% of baseline in responders to immunotherapy six months after treatment and declined to 80-90% of baseline in non-responders and placebo participants (p=0.03). This difference was not present before the six-month time point.

Discussion: Our findings provide preliminary evidence that the exocrine pancreatic enzymes lipase and trypsin may be useful biomarkers of response to immunotherapy in type 1 diabetes. Further studies with larger numbers of participants are warranted.

KEYWORDS

type 1 diabetes, exocrine pancreas, immunotherapy, thymoglobulin, granulocyte colony stimulating factor, pancreatic alpha-amylase, lipase, trypsin

1 Introduction

Type 1 diabetes (T1D) is characterized by autoimmune destruction of insulin-producing β-cells within pancreatic islets. However, the underlying pathogenesis of T1D is not yet fully understood. In healthy individuals, the exocrine pancreas, which comprises upwards of >85% of pancreatic mass, increases in size, and serum exocrine enzymes lipase and trypsin trend upwards throughout childhood and early adulthood (1–3). These enzymes are produced by pancreatic acinar cells; serum levels are reduced in diseases that cause exocrine atrophy including cystic fibrosis and chronic pancreatitis and increased in states of acute exocrine tissue damage including acute pancreatitis (4–6). Serum amylase, while frequently used for the diagnosis of acute pancreatitis, is not as specific an indicator of pancreatic disease and does not have the same gradual increase over time (2–4). There has been substantial interest in the role of the exocrine pancreas in T1D development after a study within the Network for Pancreatic Organ Donors with Diabetes (nPOD) showed that pancreas weight was reduced in non-diabetic organ donors with positive islet autoantibodies (at risk for clinical T1D) and was confirmed in subsequent research (7–9). In individuals with pre-clinical and clinical stages of T1D, pancreas size and serum and stool exocrine enzymes are significantly decreased (7–13). Most recently, Mendelian randomization studies have demonstrated that increased circulating pancreatic enzymes play a protective role in T1D susceptibility (14, 15).

Despite this progress in understanding the exocrine pancreas in T1D, no study has yet evaluated the effect of immune therapy on circulating exocrine enzymes. In this era of clinically available T1D-directed immunotherapy, biomarkers delineating response to therapy, both prospectively and in the year post-treatment, are urgently needed to further our goal of treating the right patient with the right therapy at the right time in T1D progression (16). In an era of biomarker-adaptive clinical trial designs within other fields, the benefit of early identification of response to therapy is clear, with treatment guided by indicators of therapeutic response (17). Additionally, changes in serum exocrine enzymes after treatment with immunotherapy may inform hypotheses regarding the etiology of exocrine pancreatic dysfunction in the early T1D period (13, 18). If exocrine dysfunction results from autoimmune or inflammatory destruction or insulinopenia, treatment with an immunomodulatory agent that halts the autoimmune attack or prolongs native insulin production could prevent or reverse the impact on the exocrine pancreas (13).

In this study, we sought to identify longitudinal trends in circulating amylase, lipase, and trypsin before and after treatment with anti-thymocyte globulin (ATG) and pegylated granulocyte colony-stimulating factor (GCSF) in patients with new-onset T1D. We hypothesized that effective immunotherapies in new-onset T1D would improve exocrine and endocrine pancreatic function measures.

2 Materials and methods

2.1 Study population

Study participants provided written informed consent (and assent in the case of minors) before being enrolled in the trial, as approved by independent ethics committees or Institutional Review Boards (IRBs) (19). A subset of previously collected serum obtained in conjunction with a concluded multisite, three-arm, randomized, placebo-controlled, double-blind Type 1 Diabetes TrialNet trial (TN19): "Antithymocyte Globulin (ATG) and pegylated granulocyte colony-stimulating factor (GCSF) in New Onset Type 1 Diabetes" (ClinicalTrials.gov NCT02215200) was analyzed for exocrine pancreatic markers amylase, lipase and trypsin. Participants aged 12-45 years with new-onset T1D were randomized to three treatment groups: low-dose ATG (2.5 mg/kg for one dose), low-dose ATG + pegylated GCSF (2.5 mg/kg for one dose + 6 mg subcutaneously every 2 weeks for 6 doses), and placebo, as previously described (19).

Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at two years post-treatment (20). In the overall study (n=89), 42% of treated and 17% of placebo participants met this definition. Due to constraints of sample availability, we compared longitudinal serum amylase, lipase, and trypsin levels in a subset of responders to therapy (n=4-6), placebo "responders" (n=2), treated non-responders (n=16), and placebo non-responders (n=10) at baseline, two weeks, three months, and six months after treatment. A two-year sample was not available for this analysis.

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2.2 Laboratory analysis

Amylase and lipase were quantified under blinded conditions at UF Health Pathology Laboratories (Gainesville, FL) using standard clinical assays involving direct enzymatic colorimetric analysis (Beckman Coulter, Brea, CA) and enzyme-coupled colorimetric methods, respectively. The assay-specific normal serum reference ranges were 29-103 units/L for amylase and 11-82 units/L for lipase. Serum trypsin was measured under blinded conditions at ARUP Laboratories (Salt Lake City, UT) using a standard clinical radioimmunoassay (reference range 115-350 ng/mL). We utilized existing data on β-cell function and glycemia (i.e., area under the curve (AUC) C-peptide during a 2-hour mixed-meal tolerance test at baseline, three months, six months, and two years and HbA1c % at baseline) and de-identified demographic information including age at diagnosis and sex.

2.3 Statistical analysis

Data were analyzed and graphed using R Statistical Software. Data are presented as median ± interquartile range (IQR) unless otherwise noted. Kruskal-Wallis and Fisher's Exact tests were used to compare baseline demographic information and glycemic data. The percent difference from baseline was calculated for amylase, lipase, and trypsin for each individual at two weeks, three months, and six months. Unpaired t-tests compared the percent difference from baseline to 6 months between responders and non-responders to immunotherapy and between treated and placebo participants. A Mann-Whitney test compared exocrine enzyme values at baseline in responders versus non-responders to treatment. A Fisher's exact test compared the percentage of treated responders versus non-responders having exocrine enzyme values below the reference range at baseline.

3 Results

3.1 Demographics and glycemic indices

Our subpopulation had a median age of 15 years and was 44% female, which was consistent across groups (Table 1). 2-hour AUC C-peptide was higher in responders versus non-responder and placebo participants at baseline (p=0.03), six months (p=0.003), and two years (p<0.001).

3.2 Amylase

No participant had an elevated amylase above the reference range at any measured point (baseline, 2 weeks, three months, or six months). Thirty-five percent of amylase values were below the reference range at baseline (Table 2). There were no significant

TABLE 1 Participant demographics and characteristics.

	Responders (n=4-6; 67% ATG-treated)	Non-responders (n=16; 62.5% ATG-treated)	Placebo "Responders" (n=2)	Placebo Non-responders (n=10)	Overall (n=34)	P-value
Age (years)	13.5 (13,17)	15.5 (14,16)	19 (15,23)	16 (15,18)	15 (13,17.3)	0.33
Sex (F,%)	50%	50%	50%	30%	44%	0.65
Baseline HbA1c (%)	6.4 (6,7.1)	7.6 (7.3,8.6)	8.1 (7.6,8.6)	7.5 (6.7,9)	7.5 (6.9,8.6)	0.1
Baseline AUC C-peptide (nmol/L)	0.65 (0.31,0.67)	0.28 (0.2,0.39)	0.24 (0.18,0.29)	0.41 (0.34,0.48)	0.33 (0.24,0.48)	0.03
6 mo. AUC C-peptide (nmol/L)	0.61 (0.41,0.72)	0.18 (0.13,0.27)	0.25 (0.23,0.28)	0.25 (0.19,0.3)	0.25 (0.15,0.34)	0.003
2 yr. AUC C-peptide (nmol/L)	0.48 (0.39,0.58)	0.1 (0.06,0.11)	0.19 (0.12,0.26)	0.13 (0.07,0.17)	0.12 (0.07,0.28)	<0.001

Values reported in median (interquartile range) unless otherwise noted.
Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at 2 years post-treatment. Participants were treated with either low-dose anti-thymocyte globulin (ATG), low-dose ATG + pegylated granulocyte colony-stimulating factor (GCSF), or placebo.

TABLE 2 Amylase values at baseline and 6 months.

	Responders (n=6; 67% ATG-treated)		Non-responders (n=16; 62.5% ATG-treated)		Placebo (n=12)		Overall (n=34)	
	Amylase (U/L)	% Below RR	Amylase (U/L)	% Below RR	Amylase (U/L)	% Below RR	Amylase (U/L)	% Below RR
Baseline	32.8 (28, 34.9)	33%	32.7 (24.1, 42.5)	38%	30.9 (27.8, 41.8)	33%	31.6 (26.7, 38.6)	35%
6 months	31.2 (26.2, 40.7)	50%	33.5 (23.9, 41.8)	44%	31.6 (24.7, 40.8)	33%	32.7 (25.5, 41.3)	41%

Values reported in median (interquartile range) unless otherwise noted. RR, reference range.
Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at 96 weeks post-treatment. Participants were treated with either low-dose anti-thymocyte globulin (ATG), low-dose ATG + pegylated granulocyte colony-stimulating factor (GCSF), or placebo. The reference range for amylase was 29-103 U/L.

trends in longitudinal amylase values post-treatment (Figure 1). There was no statistical difference between baseline amylase values (p=0.91) or the percentage of participants with an amylase value below the reference range at baseline among responders versus non-responders to immunotherapy (p=1.0). Responder (n=6) six-month amylase levels were 104% of baseline versus placebo "responders" (n=2) at 102%, and placebo non-responders (n=10) and treated non-responders (n=16) at 88% and 97% of baseline (p=0.86 for responders versus non-responders to immunotherapy; p=0.26 for treated versus placebo participants).

3.3 Lipase

One ATG-only treated non-responder participant had a lipase level slightly above the reference range at three months (88 U/L, RR

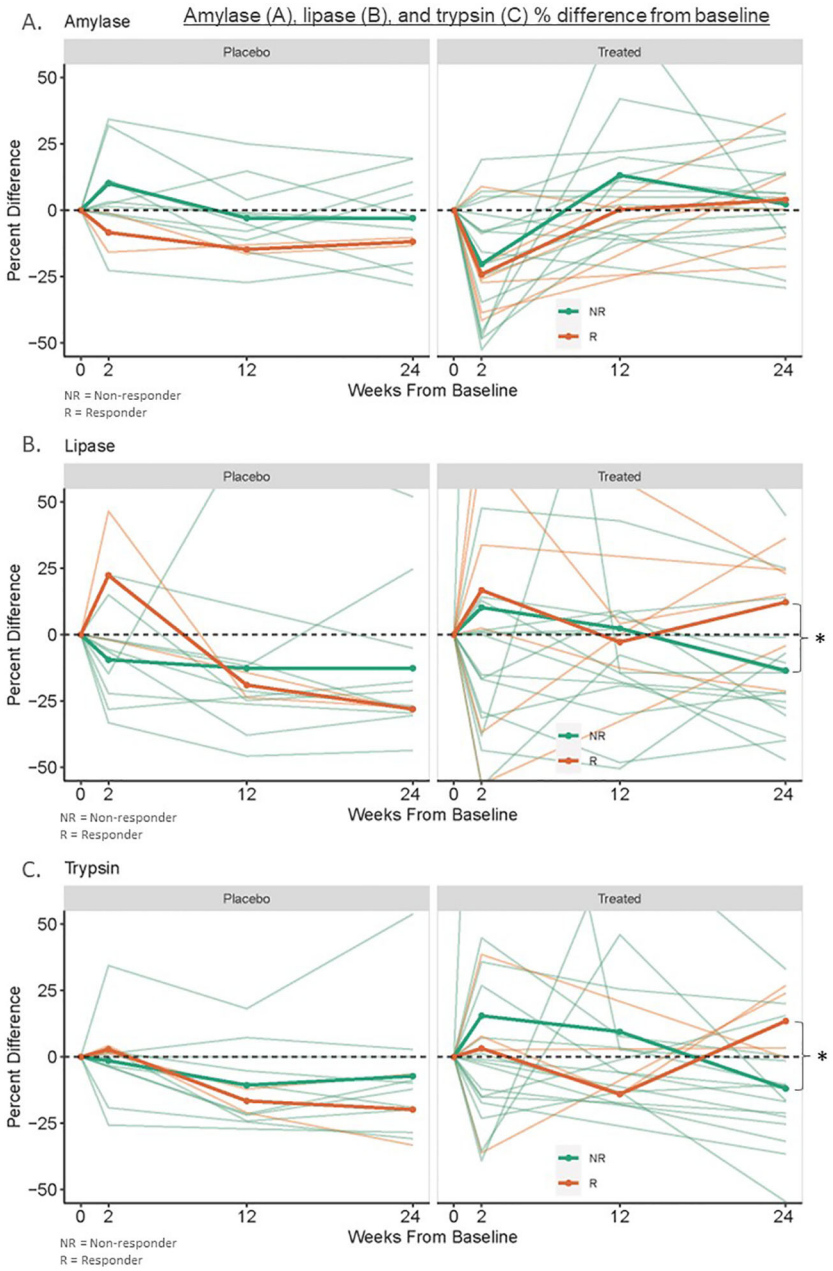

FIGURE 1
Amylase **(A)**, lipase **(B)**, and trypsin **(C)** % difference from baseline. This was measured in banked serum of new-onset type 1 diabetes treated responders (n=4-6), treated non-responders (n=16), placebo "responders" (n=2), and placebo non-responders (n=10) to therapy with ATG or ATG/GCSF. Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at 2 years post-treatment. *Treated responders had 6-month lipase and trypsin levels 116% and 115% of baseline while non-responders had levels 80% and 91% of baseline respectively (p=0.03).

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11-82 U/L); no other participants had an elevated lipase level at any measured point. Thirty-five percent of lipase values were below the reference range at baseline (Table 3). Baseline lipase values were lower but not statistically different in responders versus non-responders (p=0.08). Similarly, a greater percentage of responders had lipase levels below the reference range compared to non-responders, but this was not statistically significant (p=0.18). Responder (n=6) 6-month lipase levels were higher at 116% of baseline compared to placebo "responders" (n=2), placebo non-responders (n=10), and treated non-responders (n=16) which were at 72%, 90%, and 80% of baseline, respectively (p=0.03 for responders versus non-responders to immunotherapy; p=0.36 for treated versus placebo participants) (Figure 1).

3.4 Trypsin

Two responders did not have sufficient serum quantity to run trypsin levels, so four responder participants were analyzed. Two ATG-only treated non-responder participants and one placebo non-responder had trypsin levels above the reference range at various times (median of these participants 389 ng/mL, IQR 222-488, RR 115-350 ng/mL). Only one trypsin level at three months in an ATG-treated non-responder reached a cutoff potentially concerning for acute pancreatitis (751 ng/mL) (6), however, their concurrently measured amylase and lipase values were reassuring (68 U/L (RR 29-103) and 88 U/L (RR 11-82) respectively). Sixteen percent of trypsin values were below the reference range at baseline (Table 4). Baseline trypsin levels in responders to immunotherapy were lower than in non-responders, but this was not statistically significant (p=0.3). There was no difference in the percentage of participants with a trypsin level below the reference range at baseline among responders versus non-responders to immunotherapy (p=0.5). Responders to immunotherapy (n=4) had 6-month trypsin levels at 115% of baseline vs placebo "responders" (n=2), placebo (n=10), and non-responders (n=16) at 85%, 91%, and 91% of baseline respectively (p=0.03 for responders versus non-responders to immunotherapy; p=0.78 for treated versus placebo participants) (Figure 1).

4 Discussion

Our study suggests that two serum exocrine enzymes, lipase and trypsin, may be useful as biomarkers of immunotherapy responses in patients with new-onset T1D. In our study, six months after treatment, lipase and trypsin increased to 115% of baseline in new-onset T1D responders to immunotherapy but declined to 80-90% of baseline in non-responders and placebo participants. This difference was not present before the six-month time point. This is consistent with what has been reported regarding C-peptide differences after T1D immunotherapy, where a significant treatment effect predicting long-term C-peptide preservation is not seen until six months post-therapy (21). Additionally, in our study, no differences were seen in longitudinal amylase values. This may be due to the higher specificity of lipase and trypsin for pancreatic pathology and fits with previous studies showing decreases in lipase and trypsin along the continuum of early T1D progression with amylase only reduced in clinical disease (2). Baseline levels of lipase and trypsin tended to be lower in responders to treatment versus non-responders; however, these

TABLE 3 Lipase values at baseline and 6 months.

	Responders (n=6; 67% ATG-treated)		Non-responders (n=16; 62.5% ATG-treated)		Placebo (n=12)		Overall (n=34)	
	Lipase (U/L)	% Below RR	Lipase (U/L)	% Below RR	Lipase (U/L)	% Below RR	Lipase (U/L)	% Below RR
Baseline	9.2 (8, 14.4)	67%	15.3 (9.9, 19.8)	31%	13.6 (11.6, 16.9)	25%	14.2 (9.9, 17.7)	35%
6 months	10 (9.2, 16.6)	33%	11.4 (9.1, 16.6)	44%	11.6 (9.4, 14.3)	42%	11.2 (9.2, 15.8)	47%

Values reported in median (interquartile range) unless otherwise noted. RR, reference range.
Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at 96 weeks post-treatment. Participants were treated with either low-dose anti-thymocyte globulin (ATG), low-dose ATG + pegylated granulocyte colony-stimulating factor (GCSF), or placebo. The reference range for lipase was 11-82 U/L.

TABLE 4 Trypsin values at baseline and 6 months.

	Responders (n=4; 50% ATG-treated)		Non-responders (n=16; 62.5% ATG-treated)		Placebo (n=12)		Overall (n=32)	
	Trypsin (ng/mL)	% Below RR	Trypsin (ng/mL)	% Below RR	Trypsin (ng/mL)	% Below RR	Trypsin (ng/mL)	% Below RR
Baseline	132.6 (123.1, 161.9)	25%	153.8 (136, 187)	13%	144.3 (128.1, 212.5)	17%	145.4 (129, 196)	16%
6 months	139.5 (134.7, 191.7)	0%	123.6 (110.3, 176)	44%	132.5 (103.5, 218.1)	42%	133.1 (111.7, 190.2)	34%

Values reported in median (interquartile range) unless otherwise noted. RR, reference range.
Responders to immunotherapy were defined as participants with at least 60% of baseline area under the curve (AUC) C-peptide levels after a 2-hour mixed meal tolerance test (MMTT) at 96 weeks post-treatment. Participants were treated with either low-dose anti-thymocyte globulin (ATG), low-dose ATG + pegylated granulocyte colony-stimulating factor (GCSF), or placebo. The reference range for trypsin was 115-350 ng/mL.

variations were not significant in this small sample. Thirty-five percent of new-onset T1D participants had low amylase and lipase levels, and 16% had low trypsin levels at baseline, which is in agreement with previous studies (2, 11). Only one participant had an elevated trypsin level more than twice the upper limit of normal three months after receiving ATG, with concurrently measured amylase and lipase levels not consistent with acute pancreatitis (22). As the two week post-treatment value in this participant was within the normal reference range, this was very unlikely to be related to treatment (23). Our findings were limited by the small sample size and analysis specific to treatment with ATG and ATG/GCSF; further studies with larger numbers of participants are warranted to determine their predictive utility.

Six-month differences in lipase and trypsin from baseline may be beneficial for early identification of response to T1D immunotherapy. These serum markers should be further evaluated both individually and in combination with immune signatures and other differentiating serologic features. If our findings are replicated in larger populations, the measurement of serum exocrine enzymes could be part of a broader T1D immunotherapy individualized treatment strategy (24). In this era of emerging immunotherapeutic options to treat T1D, the early identification of response to therapy could allow for individualized decision-making regarding retreatment or treatment with another therapy and could inform adaptive trial designs (17). Additionally, possibly lower baseline values of lipase and trypsin in responders to ATG or ATG/GCSF should be further explored. A baseline signature predicting response to immunotherapy could be beneficial for improving clinical trial recruitment design and developing future individualized treatment plans (16).

This study did not evaluate other exocrine biomarkers previously identified as abnormal in pre-clinical T1D, including fecal elastase, serum carboxypeptidase A1 and chymotrypsinogen B1, and pancreas volume by MRI (7–11, 14, 15). These markers, along with a more comprehensive analysis of exocrine pancreatic protein expression, could also be helpful in evaluating response pre- and post-T1D immunotherapy. However, circulating serum lipase and trypsin have the advantage of being clinically available and easily collected along with other risk indices with low cost and high compliance.

While reduced exocrine pancreatic function at T1D disease onset has been well established, the mechanism remains largely unknown (13). The normal increase in lipase and trypsin in responders to immunotherapy could be due to a direct improvement in the autoimmune process influencing exocrine function or an indirect effect from improved β-cell function. Further experiments in preclinical models, and imaging to measure pancreas volume in future clinical studies, would be needed to determine whether this effect is mediated by an increase in pancreatic size, transcription of exocrine enzymes or prevention of their degradation, or increased release into the circulation. However, it was interesting that baseline levels of lipase and trypsin were not higher and were possibly lower in responders to immunotherapy. We had expected that the relatively higher baseline β-cell function in these individuals would be correlated with improved baseline exocrine serologic markers since many hypothesize that insulinopenia is a significant contributor to exocrine insufficiency in T1D (18). This finding should be further evaluated in a larger cohort. Our findings provide preliminary evidence that exocrine enzymes may be valuable biomarkers of response to immunotherapy in T1D and should drive future lines of inquiry in this novel and yet unexplored area.

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 humans were approved by University of Florida Institutional Review Board. 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

BB: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing, Visualization. SG: Investigation, Writing – original draft, Writing – review & editing, Data curation. RB: Formal Analysis, Methodology, Visualization, Writing – review & editing. KM: Data curation, Investigation, Methodology, Project administration, Resources, Writing – review & editing. MC: Funding acquisition, Supervision, Writing – review & editing. CW: Conceptualization, Resources, Supervision, Writing – review & editing. LJ: Resources, Writing – review & editing. MA: Project administration, Resources, Supervision, Writing – review & editing. MH: Conceptualization, Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. DS: Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Supervision, 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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Perfluorinated compounds linked to central precocious puberty in girls during COVID-19: an untargeted metabolomics study

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Background and objective: The incidence of central precocious puberty (CPP) in girls increased significantly during the COVID-19 pandemic. This study aimed to explore the impact of perfluorinated endocrine disruptors on CPP through metabolomics analysis in girls from Hainan Province, China.

Methods: Serum samples from 100 girls with CPP and 100 healthy controls were collected. Untargeted metabolomics profiling was performed using ultra-high performance liquid chromatography coupled with quadrupole-Exactive Orbitrap mass spectrometry (UHPLC-Q-Exactive-Orbitrap-MS). Differentially expressed metabolites (DEMs) were screened, and pathway enrichment analysis was conducted.

Results: Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) revealed distinct metabolic profiles between the CPP and control groups. A total of 511 metabolites were identified, including 296 up-regulated DEMs and 255 down-regulated DEMs. Three perfluorinated compounds—PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated—were significantly upregulated in the CPP group. KEGG pathway enrichment analysis suggested the involvement of multiple pathways in the CPP process regulated by these compounds.

Conclusions: Perfluorinated compounds may promote CPP in girls by interfering with various pathways and affecting the hypothalamic-pituitary-gonadal axis function. This study highlighted the need for further research and public health measures to address environmental endocrine disruptors.

KEYWORDS

central precocious puberty, metabolomics, endocrine disruptors, perfluorinated compounds, children

1 Introduction

Central Precocious Puberty (CPP) is a common and complex endocrine disorder affecting an increasing number of children worldwide (1). Recent years have witnessed a significant surge in CPP incidence, particularly during the COVID-19 pandemic (2). This phenomenon has gathered widespread attention in the medical community, yet the precise underlying causes remain elusive (3). CPP not only leads to premature development of secondary sexual characteristics but also potentially exerts long-term adverse effects on children's height development, psychological wellbeing, and social adaptability (1). Given the limited understanding of CPP etiology, an in-depth investigation of its pathogenesis and exploration of potential environmental factors, such as specific dietary compounds, are crucial. These efforts hold significant clinical importance for developing effective prevention strategies and improving patient outcomes.

Metabolomics is a discipline that studies the types, structures, quantities, changes, and functions of all metabolites within an organism. It can provide information about the metabolic state, activity, and regulatory mechanisms of metabolic pathways. Metabolomics can be used to investigate disease mechanisms, diagnose and predict disease risk, and assess the effects of drug treatments. Previous studies have utilized metabolomics approaches to investigate pubertal development in children. For instance, Fang et al. have found that pathways such as fatty acid synthesis, and gonadotropin-releasing hormone (GnRH) are closely associated with pubertal development based on an animal and clinical study (4). Wu et al. utilized a cross-platform metabolomics approach using nuclear magnetic resonance (NMR) and demonstrated that endocrine disruption caused by perfluorinated compound (PFC) exposure directly or indirectly drives metabolic changes and forms a global metabolic network disturbance in CPP (5). Therefore, metabolomics is a well-established method for studying precocious puberty in children. Finding reliable biomarkers through this approach has potential value.

This study aims to investigate the metabolomics of central precocious puberty in girls in Hainan Province, China, during the COVID-19 pandemic. Based on untargeted metabolomics, this study screened for differentially expressed metabolites (DEMs). Focusing on perfluorinated endocrine disruptors, the study analyzed the associated metabolites and performed pathway enrichment analysis to provide new insights on central precocious puberty in girls.

Abbreviations: ABC, ATP-Binding Cassette; CPP, Central Precocious Puberty; DEHP, Di(2-ethylhexyl)phthalate; DEMs, Differentially Expressed Metabolites; GnRH, Gonadotropin-Releasing Hormone; LH, Luteinizing Hormone; FSH, Follicle-stimulating Hormone; HPG, Hypothalamic-Pituitary-Gonadal; NMR, Nuclear Magnetic Resonance; LC–MS/MS, Liquid Chromatography–Tandem Mass Spectrometry; PCA, Principal Component Analysis; PFCs, Perfluorinated Compounds; PFOH, Perfluoroalkyl Alcohol; PFSM, Perfluoroalkyl Sulfonamide Methyl; PLS-DA, Partial Least Squares Discriminant Analysis; UHPLC, Ultra-High Performance Liquid Chromatography.

2 Materials and methods

2.1 Subjects

This case-control study included 100 serum samples from girls with CPP who were hospitalized and diagnosed in the Department of Genetic Metabolism and Endocrinology at Hainan Women and Children's Medical Center from January 2020 to October 2022. These samples were used as the case group (CC group). During the same period, 100 serum samples were collected from healthy participants as the control group (NN group); this group exhibited normal development and were free from any diseases. There were no statistically significant differences in age and BMI between the two groups (Table 1). This study was approved by the Medical Ethics Committee of Hainan Women and Children's Medical Center [Approval No. (2020-(010)]. Samples and clinical information were collected after obtaining informed consent from the patients' guardians.

2.2 Inclusion and exclusion criteria

2.2.1 Inclusion criteria

Case group inclusion criteria: age < 11 years, female, diagnostic criteria for central precocious puberty (1).

2.2.2 Exclusion criteria

The following subjects were excluded: those with significant clinical symptoms, severe underlying diseases, recent infections (within six months), recent antibiotic use (within three months), chronic gastrointestinal issues or surgeries, glucose metabolism disorders, or autoimmune connective tissue diseases.

2.3 Sample collection and clinical data collection

Samples from both the case group and the control group were collected by nurses from the Department of Genetic Metabolism and Endocrinology at Hainan Women and Children's Medical Center. Blood was collected in centrifuge tubes and allowed to

TABLE 1 Feature of included cases.

Features	NN (n=100)	CC(n=100)	P-value
Age (Year)	8.15 ± 0.50	8.20 ± 0.68	0.49
Height (cm)	127.85 ± 4.97	133.62 ± 7.02	<0.001
Weight (Kg)	28.44 ± 4.96	31.44 ± 6.59	<0.001
BMI (kg/m^2)	17.33 ± 2.35	17.48 ± 2.64	0.66
Basic LH(IU/L)	NA	4.28 ± 4.15	NA
LH^{Peak}/ FSH^{Peak} (Ratio)	NA	1.46 ± 0.93	NA
Bone Age (Year)	NA	9.91 ± 1.17	NA

clot and separate by standing at 37°C (or room temperature) for 1 hour. The samples were then centrifuged at 3000 g for 10 minutes at room temperature. The supernatant (1 ml) was aliquoted into 1.5 mL centrifuge tubes and stored at -80°C. Once all the samples were collected, they were used for serum metabolomics testing. Basic information and examination data were also collected from the control and case groups; they include gender, age, height, weight, BMI, bone age, basal LH value, and the LH peak/FSH peak ratio from the GnRHa stimulation test, The dietary habits of the study participants were analyzed and summarized in Table 2.

2.4 Sample preparation and quality control

The protocol outlined the preparation of samples for metabolic profiling involving LC-MS analysis. A 100 µL sample was mixed with 400 µL methanol/acetonitrile (1:1 v/v) via vortexing, then sonicated in ice baths for 1 hour to ensure thorough extraction. Afterward, it was incubated at -20°C for 1 hour and centrifuged at 4°C for 20 minutes at 14,000 g to separate and collect supernatants. These were then dried under vacuum. Quality control (QC) samples, created by pooling aliquots from all samples, were processed similarly for data normalization. Dried extracts were reconstituted in 50% acetonitrile, filtered through a 0.22 µm filter, and stored in 2 mL HPLC vials at -80°C until analysis.

2.5 LC–MS/MS analysis and data processing

The UHPLC-MS/MS analysis for metabolomics profiling was conducted using a UPLC-ESI-Q-Orbitrap-MS system involving a Shimadzu Nexera X2 LC-30AD coupled with a Q-Exactive Plus. Separation was achieved with an ACQUITY UPLC® HSS T3 column (2.1×100 mm, 1.8µm) at a flow rate of 0.3 mL/min, using 0.1% formic acid in water and 100% acetonitrile as mobile phases. The gradient profile lasted 15.1 minutes, including linear increases in buffer B to 100% and a re-equilibration period. HESI source conditions included a spray voltage of 3.8kv (positive) and 3.2kv (negative), among others. Full MS scans were acquired at 70,000 resolution, MS/MS scans at 17,500, with specified injection times and collision energies. QC samples were injected every six samples. Data was processed using MS-DIAL, with specific criteria for metabolite identification and feature selection, ensuring data quality and reliability.

2.6 Multivariate statistical analysis and KEGG enrichment analysis

The multivariate statistical analysis for metabolomics data was conducted using R version 4.0.3 and relevant R packages. The data underwent Pareto scaling for mean-centered normalization. Key statistical approaches included principal component analysis (PCA), orthogonal partial least-square discriminant analysis (OPLS-DA), and partial least-square discriminant analysis (PLS-DA). These models were evaluated for overfitting using permutation tests and they were assessed based on R2X (cumulative), R2Y (cumulative), and Q2 (cumulative) values, with perfect models having values close to 1. OPLS-DA helped identify discriminating metabolites via the variable importance on projection (VIP) score, with values greater than 1 deemed significant.

Significant metabolites were determined with VIP scores over 1.0 and a p-value less than 0.05 from a two-tailed Student's t-test. ANOVA was used for multiple group analyses, and fold changes were calculated between classes. Identified metabolites underwent cluster analysis using R.

For pathway analysis, KEGG enrichment analysis was performed on differential metabolites to identify disrupted biological pathways, using the KEGG database. A Fisher's exact test was employed for evaluating pathway enrichment, and results were adjusted for multiple testing using FDR correction, with pathways considered significant if $p < 0.05$.

2.7 Pathway analysis of endocrine-disrupting compounds

By analyzing the correlation between metabolites and endocrine-disrupting compounds (PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated) in each sample, with a correlation coefficient of 0.5 as the threshold, metabolites that were correlated with endocrine-disrupting compounds were obtained (Supplementary File S1). To visualize the results, a Sankey diagram was employed to effectively illustrate the complex relationships and

TABLE 2 Baseline characteristics of the dietary survey.

Variables	CC (n=100)	NN (n=100)	χ2/t	P value
Puffed snacks intake (%)			57.2	<0.001
None	2	16		
Occasional	45	78		
Often	46	5		
Every day	7	1		
fried foods intake (%)			23.3	<0.001
None	8	20		
Occasional	67	77		
Often	22	3		
Every day	3	0		
canned foods intake (%)			15.6	0.001
None	5	8		
Occasional	49	70		
Often	40	22		
Every day	6	0		

flow patterns among the identified metabolic pathways. The Sankey diagram was generated using the pyecharts library in the R programming language.

2.8 Data analysis

In this study, data was shown in Mean +/- SD. Data screening and processing were performed using SPSS 23.0 software, including fold change analysis, chi-square test, and t-tests. An OPLS-DA model was used to obtain variable importance for projection (VIP) values. Metabolites with VIP > 1 were considered preliminary differential metabolites between groups. T-tests further verified the significance, selecting metabolites with VIP > 1 and p-value < 0.05 as significant differential metabolites.

3 Results

3.1 Principal component analysis

Figure 1 presented the PCA of all samples, including QCs. The scatter plots of samples from both the CC and NN groups were within the 95% confidence interval, indicating overall stable quality control. Additionally, the CC and NN group samples were maximally separated, suggesting that the analytical equipment used was stable and the experimental data were reliable.

3.2 Partial least squares discriminant analysis

In the PLS-DA, the intercepts of R2 and Q2 are 0.98 and 0.97, respectively (Figure 2A). The permutation analysis revealed a Q2 value less than 0 (Q2=-1.533), indicating no overfitting in the model, thus confirming its reliability and effectiveness (Figure 2B).

3.3 Differential metabolites and pathway analysis

A total of 551 metabolites were screened (Supplementary File S2; Figure 3A). Among them, 296 metabolites—including Prolyihydroxyproline, Inosine, and Epoxygermacrone—were upregulated in the CC group, while 255 metabolites—such as Hymecromene, Vasicinone, and Glutaric acid—were downregulated in the CC group (Figure 3B). Figure 3C displays the significantly enriched (p<0.05) SMPDB pathway bubble chart. The x-axis represented the percentage of matched differential metabolites while the y-axis indicated the negative logarithm transformation of the P-value. The bubble fill color ranged from dark red to light, representing increasing P-values and decreasing significance. The results suggested potential associations between the differential metabolites and pathways such as the Urea cycle, Ammonia recycling, Aspartate Metabolism, and Methylhistidine Metabolism (Figure 3C).

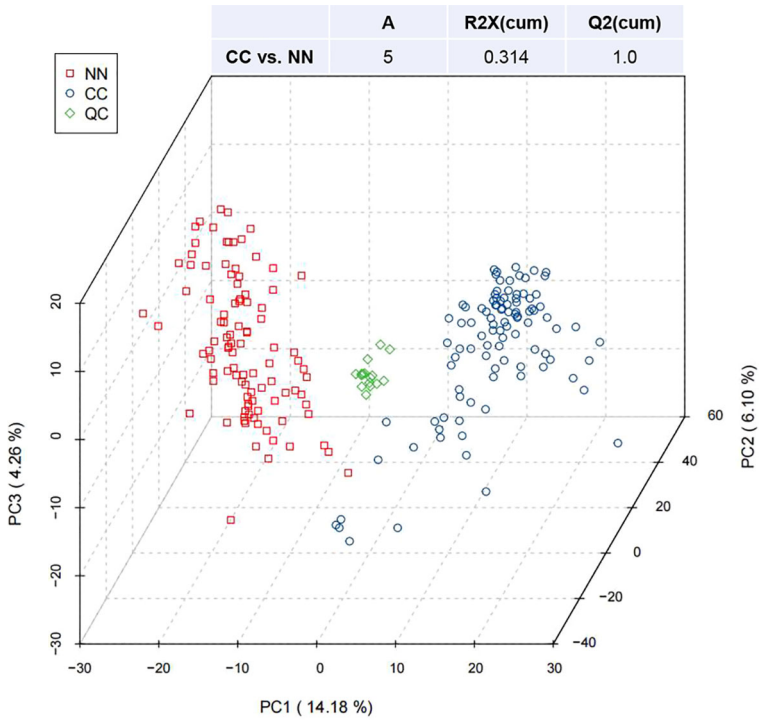

FIGURE 1
Partial least squares discriminant analysis (PLS-DA) of metabolites in the CC and NN groups.

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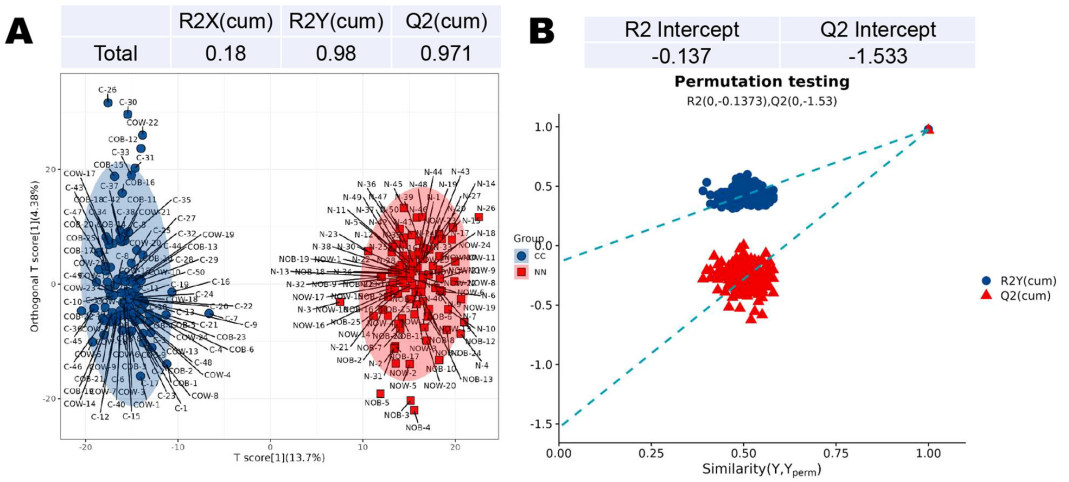

FIGURE 2
Partial least squares discriminant analysis (PLS-DA) of metabolites in the CC and NN groups. **(A)** The intercepts of R2 and Q2 were 0.98 and 0.97, respectively. **(B)** Permutation analysis revealed a Q2 value less than 0 (Q2=-1.533), indicating no overfitting in the model.

3.4 Endocrine-disrupting compounds analysis

Perfluorinated compounds and phthalates have been shown to disrupt normal endocrine function. In this study, we focused on 10 disruptors, including Monomethyl phthalate. The analysis revealed that 7 compounds—namely Monomethyl phthalate, Dibutyl phthalate, Mono(2-ethyl-5-hydroxyhexyl) phthalate, Diethyl phthalate, Dioctyl phthalate, PFOH-perfluoroalkyl_alcohol, and DEHP—were downregulated in the CC group. In contrast, 3 compounds—including PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated—were significantly upregulated in the CC group (Figure 4A). Furthermore, we performed a relative abundance analysis of perfluorinated compounds (PFOH-perfluoroalkyl_alcohol, PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-

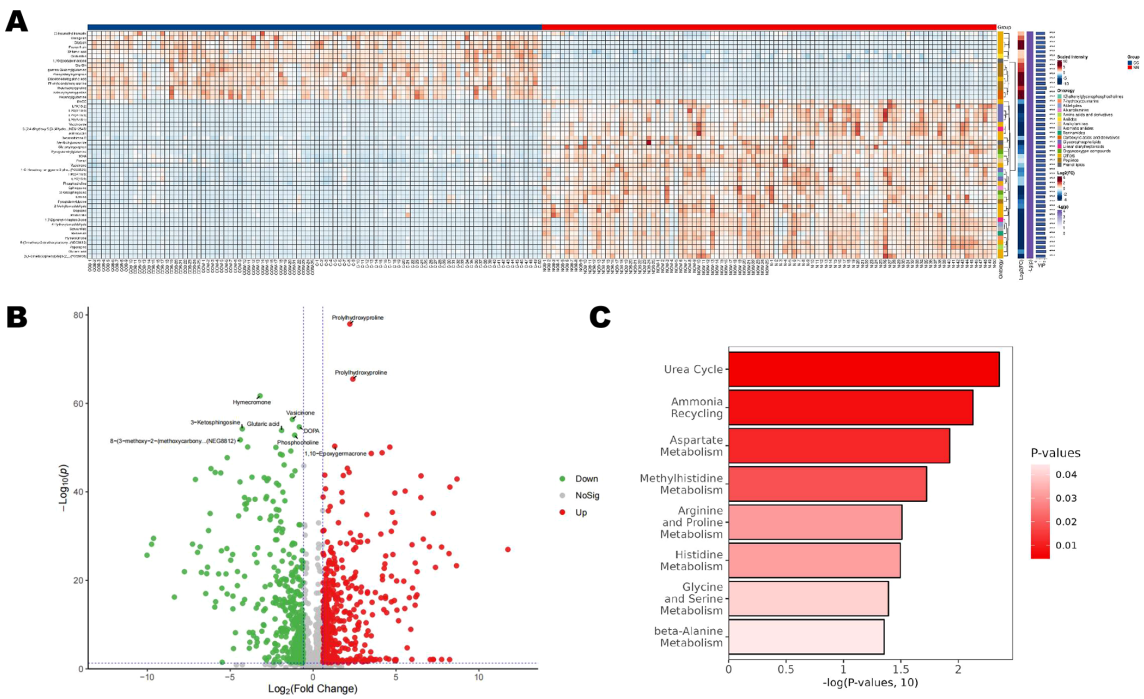

FIGURE 3
Differential metabolites and pathway analysis between the CC and NN groups. **(A)** A total of 551 metabolites were identified **(B)**, including 296 upregulated metabolites, and 255 downregulated metabolites were in the CC group. **(C)** SMPDB pathway bubble chart displaying the significantly enriched pathways (p<0.05).

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unsaturated), and the results are shown in Figure 4B. Specifically, PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated were significantly upregulated in the CC group, while PFOH-perfluoroalkyl_alcohol was significantly downregulated in the CC group (Figure 4B).

3.5 Pathway enrichment analysis of endocrine-disrupting compounds

To elucidate the metabolic pathways involved in the presence of endocrine-disrupting chemicals (PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated), KEGG analysis was performed based on the metabolites associated with these three endocrine disruptors. As shown in Figure 5, Environmental Information Processing (including ABC transporters and Neuroactive ligand-receptor interaction), Genetic Information Processing (including Aminoacyl-tRNA biosynthesis), Human Diseases (including Central carbon metabolism in cancer), Metabolism (including D-Amino acid metabolism, Phenylalanine metabolism, Arginine biosynthesis, Alanine, aspartate and glutamate metabolism, Phenylalanine, tyrosine and tryptophan biosynthesis, Glycine, serine and threonine metabolism, Taurine and hypotaurine metabolism, and 2-Oxocarboxylic acid metabolism), and Organismal System (including Protein digestion and absorption and Mineral absorption) may be involved in the central precocious puberty process regulated by the three endocrine-disrupting chemicals, PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated.

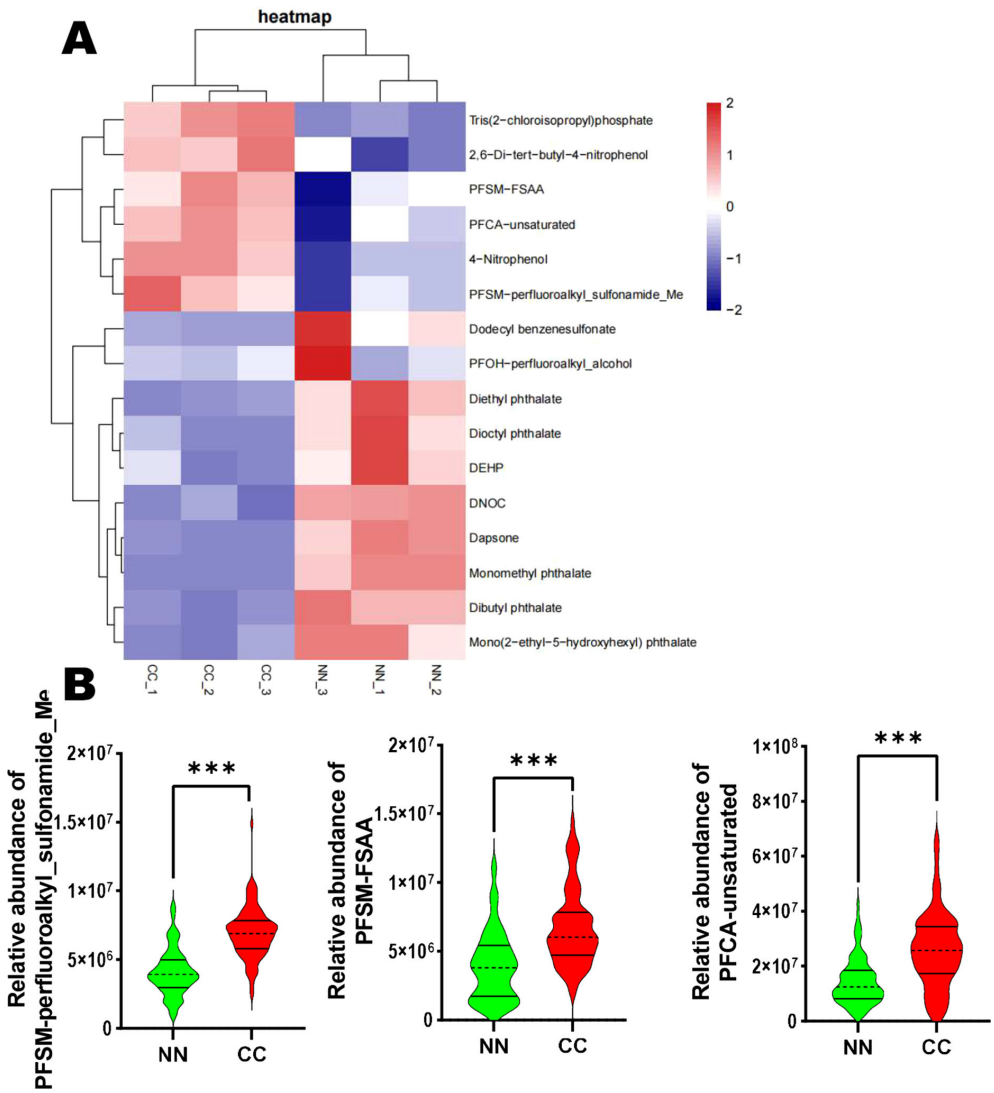

FIGURE 4
Endocrine-disrupting compounds analysis in the CC and NN groups. **(A)** Among the 10 disruptors, 7 compounds were downregulated, while 3 compounds (PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated) were significantly upregulated in the CC group. **(B)** Relative abundance analysis of perfluorinated compounds. ***P<0.001 vs. NN group.

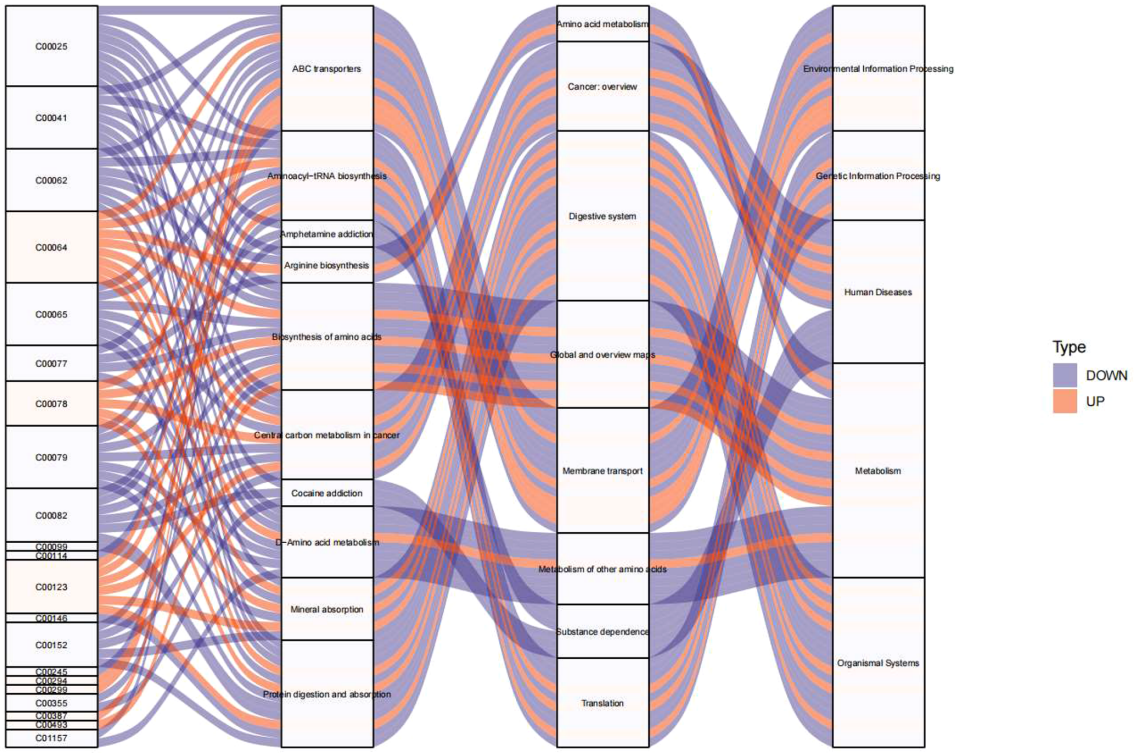

FIGURE 5
KEGG pathway enrichment analysis visualized as Sankey diagram of endocrine-disrupting compounds (PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated) associated metabolites.

4 Discussion

The COVID-19 pandemic and associated lockdown measures have led to significant changes in dietary patterns and lifestyles, which are closely related to the increased incidence of CPP (6). As an illustrative example, during the pandemic, residents' food preferences shifted, with a significant increase in the consumption of processed and canned foods (7).These foods often contain preservatives, plasticizers, and other food additives, some of which have been proven to have endocrine-disrupting effects. These additives can promote the occurrence of CPP by activating estrogen receptors and interfering with the synthesis and metabolism of sex hormones (8). Moreover, the pandemic has led to prolonged sedentary time and reduced physical activity in children, which may exacerbate childhood obesity (9). The present study employed an untargeted metabolomics approach to analyze the association between precocious puberty in girls and endocrine-disrupting chemicals in Hainan Province. The aim was to reveal potential risks for the development of precocious puberty in girls and provide new insights and strategies for prevention and intervention insofar as this condition is concerned.

Perfluorinated compounds (PFCs) are a class of synthetic chemicals containing multiple carbon-fluorine bonds; they are widely used in industrial production and daily life. However, PFCs have characteristics such as resistance to degradation, high mobility, and strong bioaccumulation. Consequently, they tend to accumulate through the food chain and ultimately enter the human body. PFSM and PFCA are a class of PFCs that can bind to estrogen receptors and mimic the effects of estrogen. Additionally, these compounds can interfere with the synthesis, transport, and metabolism of thyroid hormones, causing thyroid dysfunction (10). There is evidence to show that PFC levels in the human body are associated with factors such as drinking contaminated water, contact with food and food packaging materials, and indoor dust. Once they enter the human body, PFCs can disrupt endocrine system functions through various mechanisms (11). In addition to these mechanisms, studies have found that PFCs can also activate peroxisome proliferator-activated receptors (PPARs), affecting lipid metabolism and energy balance (12). Furthermore, numerous studies have shown that PFC exposure is associated with various health issues, such as reproductive developmental abnormalities, metabolic disorders, and neurobehavioral disorders. Wu et al. found a direct association between precocious puberty in girls and PFC exposure. Their study, based on clinical CPP patients, analyzed the serum levels of PFCs in this population and found that estradiol and prolactin were significantly associated with PFCs in CPP patients. In terms of clinical phenotype, PFCs exhibited clear characteristics of driving CPP and inducing metabolic disorders (5). Our study found associations between specific perfluorinated compounds (PFCs)—namely PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated—and CPP in children. While these findings are suggestive, further research is needed to confirm any causal relationship. Future studies should focus on more extensive environmental monitoring and population exposure assessments of

PFCs to better understand their potential impact on children's health.

Through our dietary investigation, we found that the precocious puberty group had significantly higher intake of canned foods, fried foods, and packaged puffed snacks compared to the control group. These food categories often involve packaging materials that may release perfluorinated compounds when subjected to high temperatures during processing, storage, or consumption, potentially increasing exposure risks (13). Combining our research findings that the precocious puberty group had significantly higher levels of perfluorochemicals than the control group, it suggests a close association between perfluoroalkyl sulfonamide Me (PFSM), PFSM-FSAA, PFCA-unsaturated, and central precocious puberty in children. Research has indicated that the exposure to chemicals such as phthalates and bisphenol A, widely used in plastic products, may be associated with health issues such as precocious puberty (14).Therefore, it is necessary to strengthen environmental monitoring and population exposure assessment of PFCs and take effective measures to reduce the adverse effects of PFCs on children's health. Future investigations should focus on three critical aspects: (1) comprehensive monitoring of perfluorinated compounds in the identified packaging materials, as indicated by Table 2; (2) rigorous validation of the putative role of these compounds in accelerating pubertal onset; and (3) elucidation of the molecular mechanisms underlying their potential effects on precocious puberty. These targeted research directions will be instrumental in advancing our understanding of the complex relationship between perfluorinated compound exposure and central precocious puberty in children.

Multiple pathways affected by PFSM-perfluoroalkyl_sulfonamide_Me, PFSM-FSAA, and PFCA-unsaturated may be involved in the process of precocious puberty in girls. Metabolomic analysis in the present study identified three perfluorinated compounds (PFSM-FSAA, PFCA-unsaturated, and PFSM-perfluoroalkyl_sulfonamide_Me) that may potentially influence the function of the hypothalamic-pituitary-gonadal (HPG) axis and promote the occurrence of precocious puberty in girls through various pathways. These pathways include Environmental Information Processing, Genetic Information Processing, Human Diseases, Metabolism, and Organismal System. For instance, ABC transporters have the capacity to influence the hormone balance of the HPG axis by regulating the utilization of cellular steroid hormones, ultimately exerting a decisive impact on estrogen production and affecting the process of sexual maturation (15). Studies have shown that neuroactive ligands, such as gonadotropin-releasing hormone (GnRH), play a key role in the HPG axis by stimulating the pituitary gland to release gonadotropins, which in turn regulate gonadal function and hormone production (16). Zhang et al. demonstrated that the Neuroactive ligand-receptor interaction pathway was enriched in children with central precocious puberty, which is similar to the findings of the present study (17). More importantly, the three perfluorinated compounds were enriched in metabolic pathways closely related to the HPGA or central precocious puberty, including D-Amino acid metabolism, Phenylalanine metabolism, Arginine biosynthesis, and Alanine, aspartate, and glutamate metabolism. These pathways participate in the synthesis and metabolism of various neurotransmitters and neuromodulators, thereby regulating the excitability of GnRH neurons, influencing the synthesis and release of GnRH, and ultimately modulating HPG axis function and contributing to the process of precocious puberty (18). In addition to these mechanisms, these pathways can also indirectly affect HPG axis function by influencing energy metabolism and growth and development. For example, the 2-Oxocarboxylic acid metabolism pathway involves the synthesis and metabolism of key metabolic intermediates such as oxaloacetate and α-ketoglutarate, participating in the regulation of multiple central metabolic pathways, including glycolysis, the tricarboxylic acid cycle, and amino acid metabolism (19), thereby involving in the regulation of precocious puberty (20). In the present study, by analyzing the pathways associated with endocrine-disrupting chemicals, we aimed to provide insights into the mechanisms of central precocious puberty. However, this study did not perform qualitative or quantitative detection of metabolites and pathways, which requires further research and in-depth exploration.

In summary, using an untargeted metabolomics approach, this study found that three PFCs were associated with CPP in girls, suggesting that these PFCs may promote the occurrence of CPP by interfering with pathways such as environmental information processing and affecting HPG axis function. This study revealed the potential molecular mechanisms of PFCs leading to CPP, providing new insights for prevention and intervention in the case of CPP in girls. Nonetheless, the metabolomics results still need to be validated by other omics data. Additionally, the specific pathways through which PFCs affect the HPG axis require further elucidation.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Hainan Women and Children's Medical Center. 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

HL: Writing – original draft, Writing – review & editing. MX: Writing – review & editing. HL: Writing – review & editing. YC: Writing – review & editing. LL: Writing – review & editing. HL: Writing – review & editing. YH: Writing – review & editing. YR: Writing – review & editing. JX: Writing – review & editing. XH: Writing – review & editing. XH: Writing – review & editing. WX: 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2024.1491411/full#supplementary-material

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Critical appraisal of diagnostic laboratory tests in the evaluation of central precocious puberty

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Pubertal onset is characterized by reactivation of the hypothalamic-pituitary-gonadal axis resulting in pulsatile gonadotropin secretion and subsequent sex steroid production. Accurate measurements of the gonadotropins and sex steroids are essential to ensure timely diagnosis of precocious puberty, so as to determine optimal management. This review summarizes the available laboratory testing for the diagnosis of puberty, discussing the different assays used while reviewing the limitations of such testing.

KEYWORDS

puberty, precocious puberty, GnRHa, LH, FSH

Introduction

Puberty is the process through which reproductive competence is achieved. Physical characteristics associated with this process include the development of secondary sex characteristics, acceleration in height velocity, and the occurrence of menarche in women and spermatogenesis in men (1). The exact signals that initiate puberty have only recently begun to be understood and several key targets have been identified. Pubertal onset is accompanied by increased kisspeptin and neurokinin B secretion causing the gonadotropin releasing-hormone (GnRH) neurons to secrete GnRH in a pulsatile manner which then stimulates pulsatile pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) secretion (2, 3). The LH and FSH further stimulate gonadal sex steroid secretion which promotes development of secondary sex characteristics and influences hypothalamic-pituitary function via negative feedback inhibition (4, 5). In females only, a positive sex steroid feedback develops late in puberty to trigger ovulation (6). Pubertal maturation typically starts between ages 8–13 years in girls and 9–14 years in boys (7). Central precocious puberty (CPP) is associated with early maturation of the hypothalamic-pituitary-gonadal (HPG) axis with premature reactivation of the GnRH pulse generator and sequential maturation of breasts and pubic hair in females and testicular volume, penile enlargement, and pubic hair in males. A significant long-term consequence of untreated CPP is accelerated skeletal maturation, which can result in premature epiphyseal fusion and, consequently a failure to reach genetic target height range (8, 9). Effective CPP treatment could increase final adult height and improve the likelihood of achieving one's genetic target height range (10). Boys with early-onset puberty may have behavioral difficulties and poor psychological adjustment, and girls may experience increased stress from early breast development and onset of menses (11, 12). Therefore, there is a need for accurate diagnosis and identification of children with CPP to optimize treatment plans.

In the evaluation of CPP, in addition to physical examination (for evidence of testicular enlargement in boys and breast development in girls) (13, 14) or ultrasound

examination (for evidence of enlarging ovaries/uterus in girls) (15), the most important means of defining the etiology of the precocious sexual development is to determine whether the HPG axis is activated or quiescent by appropriate endocrine tests (16, 17). Those currently available to demonstrate activation of the HPG axis are measurements of one or more of the following: random serum gonadotropin and sex hormone concentrations, urinary gonadotropins, spontaneous nocturnal gonadotropin secretion, and the hormonal (gonadotropin and sex-hormone) response to stimulation with GnRH or a GnRH-agonist (GnRHa). The diagnostic cutoffs of the concentrations of these hormones have varied depending on the assay used and therefore knowledge of the various assays, their sensitivity and specificity are paramount for clinical decision making. These tests are reviewed and their value in diagnosing pubertal onset is appraised below.

Gonadotropin measurements in the diagnosis of central precocious puberty (CPP): general considerations

Until the 1980's, gonadotropins were quantitated by radioimmunoassay (RIA), a method which allowed reliable measurement of LH and FSH at well detectable (approx. 3–4 IU/L or higher for LH, lower for FSH) serum concentrations, but could not accurately quantitate the low LH levels occurring in the early stages of puberty, due to limited sensitivity and specificity of the assay in the low LH range. This caused an overlap in serum LH concentrations between prepubertal and pubertal children (18, 19). The hormonal diagnosis of puberty required, in most instances, performance of a GnRH stimulation test, in which an LH level of 5 IU/L or greater was usually considered consistent with central puberty. When sensitive and specific immunoradiometric assays (IRMA), based on a "sandwich" assay, employing two antibodies (20, 21) for the subunits of LH, were developed in the mid 80's (22), it became apparent that small nocturnal LH pulses occur in young prepubertal children. There is an increase in frequency and amplitude in the late prepubertal period, so that between late prepuberty and Tanner stage II, LH concentrations become progressively detectable also in random daytime samples, earlier in boys than in girls, as puberty advances (19, 23). Thus, measurements of serum LH concentrations by the new highly sensitive assays allowed, for the first time, the hormonal diagnosis of puberty in random LH samples (19, 24), and appeared to better reflect the LH bioactivity[1] (26, 27). The immunometric assays for LH evolved in the following years, with transition from the IRMA (functional sensitivity[2] of about 0.15–0.4 IU/L) to ultrasensitive or highly sensitive immunofluorimetric assays (IFMA) (28) and chemiluminescent (ICMA) assays (29). These new assays (functional sensitivity of 0.02–0.05 IU/L) documented the pulsatile LH secretory activity occurring in the late prepubertal and early pubertal stages accurately, and provided reference data for random LH levels in different pediatric ages and across the pubertal stages for both sexes (23, 29–33). With regard to FSH, the highly sensitive assays showed good correlation with the RIA even at lower concentrations (23), but did not improve the diagnostic yield of measuring random FSH values for the hormonal detection of puberty, due to the substantial overlap of both baseline and GnRH-stimulated levels between prepuberty and early-mid puberty. Conversely, very low FSH concentrations, measurable for the first time by immunometric assays, became a useful marker for the diagnosis of hypogonadotropic hypogonadism (34), which can be confirmed by low FSH, LH response to GnRH stimulation as well (33). We refer the reader to a review article of gonadotropin assays (35).

Although highly sensitive assays are essential to detect the early increase in LH levels at the beginning of normal, precocious or delayed puberty in random samples, most small or non-pediatric hospitals in the US, employ LH assays (such as automated ICMAs) on laboratory "platforms" used to test several other analytes. While these assays have sufficient clinical sensitivity (limit of detection 0.1 IU/L, functional sensitivity usually ~0.2 IU/L) (36, 37) to measure LH in children with mid-advanced puberty, and good specificity, they are often inadequate to detect the low LH concentrations occurring at the onset of puberty. Of note, assays of different sensitivity may be produced by the same manufacturer [for example, HS-DELFIA with an LH functional sensitivity of 0.05 IU/L (38), vs. autoDELFIA (39), with sensitivity of 0.6 IU/L] making it important to focus on the sensitivity of the assay quoted in different papers.

The other critical issue, which is rarely discussed about these assays is that of (excessive) specificity. While the monoclonal antibodies currently employed in most LH immunometric assays have been useful in assuring reproducibility of the assays over time and minimal cross reactivity with beta-hCG, the possibility of "excessive specificity" should be kept in mind as a factor that may lead to "inappropriately" low LH levels. Unlike other hormones secreted in a definite molecular form (such as T3 and T4), the glycoprotein hormones, including LH, circulate in different isoforms, related to post-translational modifications in glycosylation, sialylation and sulfation affecting the mass and content of the carbohydrate moiety, as well as the charge of the

[1]The bioactivity of LH has traditionally been measured by the rat interstitial cell testosterone (RICT)assay described by Dufau et al. (25). In this assay serum from a patient is added to suspension of Leydig cells obtained from rats (or mice in modification of the procedure), and the testosterone released in the medium is quantitated as an index of the LH biopotency.

[2]Functional sensitivity, also referred to as the limit of quantitation, is the minimal concentration at which the intra-assay coefficient of variation (CV) is <20%. The limit of detection, or "sensitivity", is usually defined as the minimal concentration above 2 SD of the 0 standard.

molecule. While changes in the carbohydrate component do not interact directly with the antibodies to LH, which are directed against the amino acid/protein epitopes, they may indirectly affect the binding of LH to the Ab in the immunoassay and to its receptor, resulting in variability of the "bioactivity*, the bioactive/immunoreactive ratio and half-life of different LH isoforms (40). A second issue related to interpretation of LH concentrations is the existence of molecular variants of LH with low immunoreactivity and high bio/immune ratio. The most common is a modification in two amino acids of the LH beta subunit (41), which is very common in Finland but may also be relatively common in other countries. Heterozygotes with this variant (about 24% of the Finnish population) show lower than average LH concentrations in most immunometric assays, and homozygotes (approximately 3% in Finland) will have very low or undetectable LH values (42). This variant appears to have normal bioactivity in adult healthy women (41), although it may be associated with a higher risk of PCOS in some women (43) and slower tempo of puberty in adolescent boys (44). This type of "invisible" or "partially visible" LH may cause puzzling clinical conundrums, with conflicting clinical and laboratory findings which may prompt complex and costly investigations (45), as shown in a vignette from our clinic below (see Box 1).

BOX 1 Case.

An 8-year-old boy was referred for early puberty. On PE his height was at the 90th percentile (vs. mid-parental height at the 40th percentile) with evidence of growth acceleration during the last year from his pediatric records, weight at the 60th percentile. Bone age was advanced at 10.5 years. General PE was unremarkable. He had early T3 genital development, with testicular volume of 6–7 ml, pubic hair was stage 2. Laboratory tests showed DHEAS of 30 ug/dl, basal and ACTH-stimulated 17-hydroxyprogesterone <120 ng/dl, total testosterone of 65 ng/dl at 9 AM, FSH of 1.2 IU/L and LH < 0.025 IU/L. A leuprolide stimulation test resulted in a peak FSH of 7.7 IU/L, persistently undetectable LH of <0.025 IU/L at multiple time points, and a 24 h stimulated Testosterone of 260 ng/dl. To exclude a laboratory error, the Leuprolide test was repeated 2 weeks later, with similar results (undetectable baseline and stimulated LH; baseline T of 54 ng/dl, 24 h stimulated testosterone of 233 ng/dl). The 60 min sample was split and sent to 2 different laboratories for the LH assay. While our usual laboratory (Lab A) reported an LH value of <0.025 as mentioned, Lab B reported a value of 5.6 IU/L, which confirmed the clinical diagnosis of CPP, determined to be idiopathic. He was treated with GnRHa with good clinical response and normalization of serum Testosterone to <10 ng/dl. We were unable to obtain genetic studies to evaluate the likely possibility of a variant LH molecule.

Key Points: Unlike the old LH RIAs, the current immunometric LH assays are sensitive and specific, allowing detection of early pubertal concentrations in unstimulated LH values in a subset of early pubertal children. However, sensitivity may be suboptimal in "platform" assays used in most hospitals. Conversely, excessive specificity, related to the characteristics of the antibodies used in the assay, or the presence of LH molecular variants with low immunoreactivity, may result in underestimation of LH values.

Baseline LH measurements for the hormonal confirmation of central precocious puberty (CPP) or delayed puberty

Random LH concentrations are commonly measured in the initial evaluation of children with either premature or delayed pubertal development. Random LH values may be higher in the early morning than at other times during the day (46), although the maximal nocturnal concentrations occur soon after the onset of sleep (33). We and others have shown that random LH concentrations can be used to detect onset of puberty in boys who are "late bloomers", as LH increases before testicular enlargement is noted (19, 47). The cutoff value considered to be "pubertal" for a random LH value measured with the current, highly sensitive (HS) assays (HS-IFMA, HS ICMA, with limit of quantitation ≤0.05 IU/L), is generally ≥0.3 IU/L (48, 49). The diagnostic sensitivity of a random LH value in the diagnosis of CPP in girls has been evaluated by different groups with variable results, ranging from <50% (50) to 100% (49), with most studies reporting intermediate sensitivity and similarly variable specificity (Table 1). Large studies are more meaningful in this regard. In a large cohort of 449 girls (38), 65% of girls with CPP and 26% of girls with "early normal puberty" (onset between 8 and 9 years) had a random LH > 2 SDS of a control group of girls, and a number of girls with pubertal response to GnRH had prepubertal basal LH values. In another study of over 150 children with CPP, 85% of girls and 97% of boys had a random HS ICMA LH ≥ 0.3 IU/L (48), and the latter subgroup had a generally more advanced pubertal stage than those with lower/undetectable LH. However, some of the girls with stage 4 breast development, and 1 post-menarcheal girl had LH <0.3 IU/L. In another large study (56), the authors noted minimal increase in serum LH concentrations between Tanner B1 and B2, and persistently low LH levels (≤0.2 IU/L) in some children up to stage B4, although this study was marred by using an automated ICMA of suboptimal sensitivity (Immulite 2000 XPi®) which, in our opinion, makes LH values ≤0.3 IU/L difficult to interpret.

The variable diagnostic sensitivity of random LH concentrations at the time of the initial assessment even when measured by highly sensitive assays, is likely related to multiple factors in different studies, in addition to the characteristics of the assay. These include the different intervals between the onset of detectable breast tissue and the hormonal measurements (Tanner B2 may last for 6 or more months), which may be affected by the patterns of referral in different clinics/countries/

TABLE 1 Sensitivity and specificity of basal and stimulated LH values in the diagnosis of puberty.

LH values (IU/L)	Method	Sensitivity (%)	Specificity (%)	Study (reference)	Comments
Unstimulated LH < 0.3 (prepubertal) ≥0.3 (pubertal)	ICMA	77–93 48	100 100	Houk et al. (51) Pasternak et al. (52) Neely et al. (49) Logan and Eugster (48) Harrington and Palmert (53) Sathasivam et al. (50)	While a pubertal highly sensitive LH can confirm a diagnosis of CPP, a prepubertal value does not refute it.
Stimulated LH > 4.9 (GnRH)	ICMA	78	79	Pasternak et al. (52)	The transition to a LH-predominant response is often a relatively late development in the clinical progression of central precocious puberty
Stimulated LH > 5 (2 h, Leuprolide)	ICMA	78	100	Sathasivam et al. (50)	
Stimulated LH > 5.5 (3 h, Leuprolide)	ECLIA	93	100	Carretto et al. (54)	
Stimulated LH > 6 (1 h, Triptorelin)	ICMA	89	91	Poomthavorn et al. (55)	

ICMA, immunochemiluminometric assay; ECLIA, electrochemiluminescence; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; CPP, central precocious puberty.

health care systems, leading to a selection bias; the different time of the day when LH was measured; preselection criteria such as a "pubertal response" to GnRHa (49); and lack of clinical follow-up to document pubertal progression in some studies. Overall, all the quoted and other papers (51, 58) suggest that, while a baseline LH ≥ 0.3 IU/L by highly sensitive IFMA or ICMA is consistent with CPP, a lower random LH value does not exclude CPP. This was clearly stated in a previous document on the use of GnRHa in children (59), and is keeping with a classical observation (based on the LH response to GnRH in the LH-RIA era), that CPP in girls develops along a continuous spectrum of clinical and hormonal changes (60). This concept is reinforced in the above mentioned recent study by Madsen et al. (56) who, by using a sensitive breast ultrasound (US) technique, noted initial breast changes of puberty approximately 2 years before the onset of palpable breast tissue (Tanner B2) and described in detail the continuum of hormonal changes through the pubertal stages. Data are scantier in boys, but it appears that LH values are higher and more frequently in the detectable range in G2 boys than B2 girls (23, 48). A recent large study in boys correlated random gonadotropin and testosterone levels to pubertal stages, assessed both clinically and by testicular sonogram (57).

Key Points: random serum LH levels ≥0.3 IU/L by highly sensitive assays suggest the hormonal onset of puberty in both sexes. With less sensitive LH assays, a higher cutoff value may need to be employed (we suggest 0.5 IU/L). It is appropriate to confirm the LH elevation with a repeat, early morning sample, if the purpose is to forgo a GnRH or GnRHa stimulation test. However, undetectable LH concentrations (with any assay) do not exclude the hormonal onset of puberty, and may occur in ~20%–35% of girls and ~5% of boys with early hormonal changes that can be detected by a stimulation test.

Nocturnal LH measurements

The measurement of spontaneous LH secretion by highly sensitive assays in blood samples, collected every 10–30 min could arguably be considered the most physiological method for detection of the early hormonal changes of puberty (31–33, 35, 61, 62). Unfortunately, the procedure is invasive, costly, requires hospitalization, and is generally unavailable outside of a research setting. We will briefly outline below the data regarding correlation of nocturnal LH levels with GnRH and GnRHa-stimulated LH levels.

Measurement of urinary gonadotropins

Measurement of urinary gonadotropins in timed urine collection by traditional RIA has been available for decades (63, 64), notwithstanding the limited sensitivity of the assay (requiring urine concentration) and reported periodic fluctuations of gonadotropin levels (65). With the advent of the immunometric assays, LH measurement in random or early morning urine samples appeared to provide sufficient sensitivity (66). Urinary gonadotropins increased with advancing age and pubertal development and were detectable in first-morning urinary void, even before physical signs of puberty (67). They were also noted to identify sex-specific gonadotropin changes during early infancy (68). Despite its minimal invasiveness, urinary gonadotropin testing has not become popular in the U.S. However, renewed interest in this procedure has been recently fostered by investigators predominantly from Asian centers (69–71).

Key Points: Urinary gonadotropins (LH particularly) provide "integrated" hormonal concentrations and are a useful adjunct for the diagnosis and treatment of CPP. They have been employed mostly in Europe and Asia, and they have not been as extensively studied as the corresponding serum measurements.

Baseline sex hormones in the diagnosis of puberty

In girls, serum estradiol (E_2) concentrations are often undetectable or very low (<15 pg/ml or <55 pmol/L) in random daytime blood samples obtained in early to mid-puberty (Breast stage 2–3). In these early pubertal girls, nocturnal pulses of estradiol occur and are sufficient to induce development of

secondary sexual characteristics, so that E2 levels can be quantitated, when measured, by a sensitive assay, during the night or in early morning samples. In this regard, it is important to briefly discuss the unextracted, competitive "platform" estradiol immunoassays commonly used in most hospitals. Even though these assays tend to correlate reasonably well (albeit with variable bias) with the reference liquid chromatography-tandem mass spectrometry (LC-TMS) assay at E2 values of ~25 pg/ml or higher, they correlate poorly with the reference method at lower E2 concentrations (72), thus providing inadequate sensitivity and specificity and misleading results at the low E2 concentrations encountered in early-mid female puberty (73). As mentioned above, the current "gold standard" for serum E2 measurement in the low range seen in (early) pubertal girls is the LC-TMS method (74, 75), with a functional sensitivity of 1–3 pg/ml. Nonetheless, competitive immunoassays which include extraction and column chromatography (76), or even solvent extraction alone (73) provide good sensitivity (~2–5 pg/ml) and adequate specificity in early female puberty in laboratories that have no access to the more expensive LC/MS/MS method.

In boys, serum testosterone (T) is secreted by the Leydig cells or derives from conversion of adrenal androgens, so that the small T increase (up to ~30 ng/dl) in early pubertal, adrenarcheal boys cannot be attributed with certainty to gonadal activation. For this reason, measurement of early increases in serum testosterone in boys has lower diagnostic sensitivity than early detection of E2 increase in girls for the diagnosis of CPP, with the understanding that sex hormone measurements should always be paired with serum LH measurements. Thus, serum T measurement by direct, unextracted assays has been used to define diurnal testosterone rhythms in boys and have shown average testosterone concentrations of ~30 ng/dl in an early morning sample in early puberty, heralded by testicular volume of 3–6 ml (77). Other clinicians have proposed a serum T level of >40 ng/dl to indicate the onset of puberty in boys (78). Nonetheless, the "gold standard" for T measurement in children is the LC-TMS method (57, 79), or an immunoassay involving manual purification steps (80), as direct testosterone assays correlate poorly with the reference method at low T concentrations (81). As an aside consideration, direct testosterone assays are truly inadequate for assessment of hyperandrogenic conditions in pubertal girls or adult women (82–84).

Lastly, high dose (>1–3 mg/day) biotin (vitamin B7) which is available as an over-the counter supplement used to strengthen nails and hair, interferes with the technical aspects of immunoassays and can lead to either falsely elevated or falsely low results when streptavidin binding is utilized in the assay detection system. Biotin does not interfere with LC-MS/MS assays (85).

Key Points: E2 levels should be measured by highly sensitive assays (LC-TMS or at least extraction methods) to detect early pubertal changes in girls. Even so, E2 may be truly undetectable or very low at stages B2 and early B3. T values >30–40 ng/dl (measured by a sensitive method, LC-TMS or at least extraction assay) are generally consistent with the onset of hormonal puberty in boys. However, the same levels and especially lower levels above prepubertal (>5–10 ng/dl) may be due to adrenarche, gonadarche or a combination thereof and not be truly diagnostic of gonadarche alone. If immunoassays (i.e., methods not based on mass spectrometry) are used and the results do not make sense clinically, use of high-dose biotin supplements should be excluded.

Inhibin B levels in the diagnosis of puberty

Inhibin B (INHB) is produced in the Sertoli cells of the testis in males, and in the granulosa cells of the ovary in females. It belongs to the transforming growth factor-β super family and regulates the synthesis and secretion of FSH in a negative feedback loop (86). In males, INHB level reflects Sertoli cell number and function and peaks shortly after birth, decreases during childhood, and then increases at puberty due to FSH stimulation. In females, INHB level is related to the number of antral follicles and reflects the ovarian response to gonadotrophins (87). Undetectable or low INHB levels are observed in boys with either congenital or acquired absence of testicular tissue whereas normal or near-normal levels are seen in cryptorchidism and disorders with preserved Sertoli cell function in spite of absence of germ cells or impaired androgen biosynthesis or action (88). While Individual studies have shown basal inhibin B to have good accuracy to predict the onset of puberty, diagnostic thresholds given by different studies are variable and overlapping. Specifically, there is considerable overlap in INHB concentrations between boys with testicular volume 1–3 ml and those with volume >4 ml (89), thus a single diagnostic cutoff for routine clinical practice is still unavailable (90). Recently, FSH stimulated INHB levels has been explored as a promising investigation for prediction of onset of puberty (91).

Key Points: INHB increases at puberty in both sexes, but has been mainly used in males (who have higher values of INHB throughout life), as a marker of puberty. It can be used as an additional hormonal parameter, in the context of a multi-hormonal evaluation, but is inadequate as an isolated maker of puberty in boys.

Stimulated LH in the diagnosis of puberty

In the US, the traditional GnRH stimulation test has been replaced by the GnRHa stimulation test since the mid 1980's, when GnRH became commercially unavailable. Different GnRHa have been used in this country, including parenteral Nafarelin (now unavailable), and, more commonly, subcutaneous (SC) aqueous Leuprolide, which has been used for decades. Triptorelin (which had limited research availability as an aqueous solution in the US) has been used mainly in other countries, and depot preparations of GnRHa have also been used for stimulation, as they release a substantial proportion of the analog rapidly, immediately after injection, resulting in FSH and LH stimulation (92, 93). After an initial study with aqueous SC nafarelin (94), our experience has been limited to aqueous SC leuprolide, which we have been using exclusively for diagnosis of pubertal disorders (95). For monitoring children treated with GnRHa for

CPP, we have monitored FSH, LH 1–3 h after stimulation test by aqueous leuprolide and, more recently, by the same Leuprolide depot preparation being used for treatment. We (50, 95) and others (96) have used a Leuprolide dose of 20 mcg/kg. Lower doses of 10 mcg/kg (97) or a single dose of 500 mcg (98) appear to achieve effective stimulation of FSH and LH secretion, but no dose-response studies are available in the 5–20 mcg/kg dose range. Triptorelin has similarly been shown to be effective (55, 99). Although there may be subtle differences in the effectiveness of different GnRHa, probably all of them at the dose commonly employed achieve (sub)maximal FSH and LH stimulation. Few studies have compared the LH response to leuprolide to the response to GnRH stimulation in the same subjects. In a comparison study with testing a few weeks apart, Ibanez (98) showed Leuprolide, 500 mcg SC to induce a LH peak which was almost twice as high as the peak induced by GnRH IV in children with advanced precocious puberty, while response to the 2 agents was similar in prepubertal children. In an analogous, small ($N = 8$), unpublished study of early-mid pubertal girls (Breast T2–T3) with CPP tested with GnRH IV (2.5 mcg/Kg) 3–5 months after undergoing a Leuprolide stimulation test, we found that SC leuprolide (y) achieved a ~1,4 times higher LH peak average than GnRH despite these patients being a few months older at the GnRH test. These limited data suggest that Leuprolide achieves at least a similar, and possibly greater stimulation of LH (measured by immunometric method) than native GnRH in children evaluated for CPP. Information regarding correlation between the peak LH to Leuprolide and the spontaneous nocturnal LH secretion is likewise limited. Following a study showing good correlation ($r = 0.83$) between nocturnal HS- IFMA LH concentrations and (very) low-dose GnRH-stimulated LH peak values (62), we performed an analogous analysis in 28 early to mid-pubertal girls with premature or early pubertal development. The study group included 25 girls with CPP (progressive on follow-up), and 3 with non-progressive exaggerated thelarche (100) (unpublished data). Of the girls with CPP, 7 [age 6.9 ± 0.5 years (mean ± SD), Δ(BA-CA) 1.6 years] were at Tanner stage B2, with LH < 0.25 IU/L in 5, 0.3–0.4 IU/L in 2 girls; baseline (extracted) E2 < 5 pg/ml in 5, 6–7 pg/ml in 2 girls]. Fifteen girls [age 6.8 ± 0.6 years, Δ(BA-CA) 2.2 years], were at stage B3, with baseline LH < 0.25 in 4, 0.25–0.5 in 5, 0.5–1.4 IU/L in 6, and E2 < 5 pg/ml in 7, 5–16 pg/ml in 8 girls). Three girls were at stage B4 [8.7 ± 0.3 years, Δ(BA-CA) 3.8 years], with baseline LH 1.1–2.7 IU/L, E2 10–25 pg/ml. Three girls with exaggerated thelarche [age 1.7 ± 0.3 years, Δ(BA-CA) 1 year] had LH < 0.25 and E2 < 5 pg/ml. In this cohort, we evaluated the relation between 12 h (8 PM to 8 AM) LH-IRMA concentrations (sampled every 30 min) and peak response to Leuprolide (20 mcg/Kg SC). The peak LH (IRMA) after leuprolide showed good correlation with the peak nocturnal LH ($r = 0.83$, $p < 0.001$, Figure 1) and the mean nocturnal LH ($r = 0.89$, $p < 0.001$). A subsequent study from the U. of Chicago, showed that the peak sleep LH concentration, measured during an approximately 3 h period after the onset of sleep, also correlated well ($r > 0.8$) with both the peak LH after Leuprolide, as well as the peak E2 after leuprolide, in normal pubertal girls (101).

While the above information is useful to validate the GnRHa stimulation test and to interpret clinical studies regarding diagnostic sensitivity and specificity of various tests for the diagnosis of CPP, most pediatric endocrinologists have been seeking a specific cutoff value for stimulated LH to diagnose

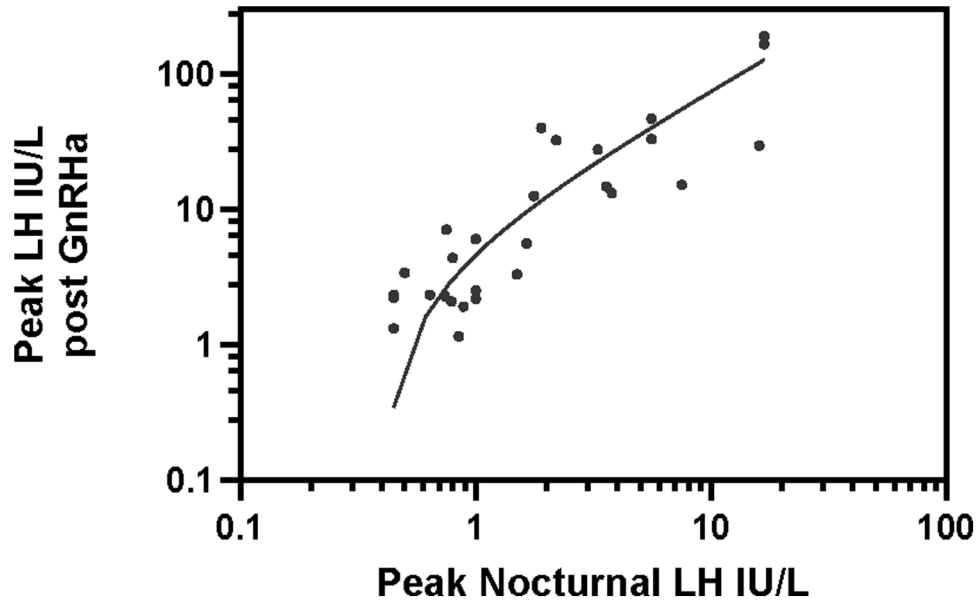

FIGURE 1
Correlation between nocturnal peak LH vs. Leuprolide stimulated peak LH. y (GnRHa stimulated peak LH) = 7.7 x −3.1 IU/L, where x = peak nocturnal LH. $r = 0.83$; $r^2 = 0.69$ $p < 0.001$. LH, luteinizing hormone.

pubertal activation, when baseline LH levels are non-diagnostic. Clinicians have continued to use a cutoff GnRH-stimulated LH value >5 IU/L for the diagnosis of precocious puberty (37), the same value traditionally used LH-RIA, without taking into consideration the differences between RIA and immunometric assays, between different immunometric assays, and the likely potency difference between GnRH and GnRHa (98). Other investigators have proposed various diagnostic threshold values for LH concentrations in the diagnosis of CPP, based on their clinical data. These cutoffs include 3.3 IU/L for IFMA vs. 4.1 for ICMA (102); 6.9 (girls), 9.6 (boys) IU/L for IFMA (39); 8.0 IU/L for ICMA (98); 6.0 IU/L for automated platform chemiluminescent assay (ECLIA) (55); 7 IU/L (IFMA); 8 IU/L (platform ECLIA) (99). Moreover, we have demonstrated that Leuprolide-stimulated LH values by IRMA (95) or HS-ICMA (50) well below the traditional value of 5 IU/L can be associated with CPP. Other investigators have noted that the diagnostic sensitivity of the leuprolide stimulation test, for a stimulated LH cutoff level of 5 IU/L, is highest when the test is prolonged to 180 min, thus allowing the LH to rise maximally (58). In our opinion, defining a universally applicable GnRHa-stimulated LH cutoff value for the diagnosis of CPP is an elusive goal, given the variability and limited standardization of the different LH assays used, the variable data arising from different populations on which proposed threshold values are based, the duration of the GnRHa tests employed, and the fact that, in the continuum of pubertal progression (103), a "low" LH response at one point in time is not necessarily predictive of the speed of future progression. Other caveats regarding interpretation of LH cutoffs to GnRHa include lower values noted in girls with obesity (104). For all these reasons, we think that, for the diagnosis of CPP in girls, the LH response to stimulation, rather than in absolute values, needs to be interpreted in conjunction with the other clinical, radiological and hormonal parameters, including the sex-hormone response to GnRHa stimulation (discussed below), and, as importantly, an observation period of 3–6 months to evaluate progression in cases that are not clear-cut. Because the LH secretion tends to occur earlier in boys than in girls, for the same degree of sexual development in normal and precocious puberty (23, 48), an LH response to GnRHa > 5 IU/L may be theoretically more universally applicable, however data on cutoff LH response to stimulation are limited for boys with CPP, due to the lower prevalence of this condition in males (105–107).

Different cut-points need to be used to interpret random LH concentrations in girls under two years of age because LH concentrations may normally be higher (following the "minipuberty" of infancy); CPP may frequently be misdiagnosed during this phase of development (108). The interpretation of LH response to GnRH or GnRHa is also difficult in these very young girls with premature sexual development as a subgroup of them with idiopathic premature thelarche may show an LH response >5 IU/L to stimulation (108). Additionally, some girls with atypical or "exaggerated" thelarche may show a robust 20–24 h Estradiol response to leuprolide stimulation (100) and still have a self-limited condition.

Key Points: The LH response to Leuprolide stimulation correlates with the response to GnRH stimulation and with nocturnal LH secretion (with the limitation that correlation studies are few). Although an LH peak >5 IU/L to stimulation has been considered the hallmark of hormonal puberty, pubertal development in girls can be seen at lower LH values, which may be due to different characteristics among LH assays, the continuum of puberty, or other factors. Thus, a universal "pubertal cutoff" for the LH response to GnRHa may be an elusive goal, at least in girls.

Sex hormone response to GnRHa in the evaluation of puberty

The original observation that GnRHa administration achieves sequential gonadotropin and estradiol secretion in girls with CPP (94, 95) was subsequently expanded to include an equivalent response in boys, adult men and women (78, 97, 109). Given the above noted difficulties in establishing a diagnostic GnRHa-stimulated LH cutoff value to diagnose precocious puberty in girls, the estradiol response to Leuprolide or other GnRHa (and the analogous testosterone response in boys) can be utilized as evidence of the activation of the entire pituitary-gonadal axis at puberty. We reported that ~20% of girls with progressive CPP undergoing Leuprolide stimulation did not achieve a predetermined "diagnostic" LH peak value of 5 IU/L by ICMA, yet they could be diagnosed by measuring a peak E2 response >50 pg/ml at 24 h (50). The usefulness of the stimulated (20–24 h post injection) E2 level for the diagnosis of CPP has been supported by other investigators (99, 110) and confirmed in our routine clinical experience (unpublished data), but not all (54). In this regard, the proposed cutoff for peak stimulated E2 value to diagnose CPP ranges from 40 pg/ml (98) to 80 pg/ml (54, 99) and a study suggested that a percentage increase of E2 (>28%) between 3 h and 24 h had the best diagnostic accuracy (110). It is likely that these differences are related both to characteristics of the E2 assay employed, and the variably advanced pubertal development in the different populations studied. For the last few decades, we have found that the ranges we employed in our original report (20–24 h E2 responses to leuprolide > 50 pg ml, by an extracted E2 assay, consistent with progressive/advanced CPP, and responses of 25–50 pg/ml consistent with early/slowly progressive CPP) (95) have correlated well with outcome, follow-up and the need for GnRHa treatment. Nonetheless, we realize that any cutoff value is somewhat arbitrary, also in consideration of the continuum of puberty discussed above for stimulated LH cutoff values.

In boys, there seem to be no consensus about cutoff GnRHa-stimulated T levels for the diagnosis of CPP, as reported series showed baseline T levels in a clear pubertal range in Tanner stage 2 (T2) boys (111), and even in T1 boys (109), thus making the stimulated T values clearly elevated and uninterpretable for diagnostic purposes. Studies of boys with delayed puberty may be more informative in this regard. Three adolescent boys with constitutional delay of puberty (Tanner 1) had an average peak T response of 65 ng/dl, significantly higher than the peak of 20 ng/dl

achieved by 8 subjects with hypogonadotropic hypogonadism (112). In a cohort of prepubertal (T1) adolescents and young adults, Lanes et al. showed a peak T response of 29 ± 12 ng/dl in 8 subjects with Gonadotropin deficiency compared to 110 ± 20 ng/dl in 14 subjects with delayed puberty (all subjects were T1), however noted overlap of T responses (as well as LH responses) between the 2 groups (96). In the absence of more definite studies, we have empirically interpreted a cutoff (20–24 h) T response ≥100 ng/dl, or an increment (delta) of >60 ng/dl above baseline, as indicative of CPP in boys, with good clinical correlation. We feel, however, that studies of the testosterone response to GnRHa in normal boys (a larger population than boys with sexual precocity) on the brink of puberty (late T1 stage) would be helpful for interpretation and validation of the cutoff T response to GnRHa for the diagnosis of CPP. While we have found that the LH response to GnRHa is generally sufficient to confirm the diagnosis of CPP in the great majority of boys for their consistent increase in LH secretion at stage T2 (23, 48), the T response can be confirmatory and useful in atypical cases of sexual precocity, as described in the vignette from our clinic (see Box 1).

Key Points: As a LH response <5 IU/L (or whatever threshold value is chosen) to GnRHa does not uncommonly occur in girls with CPP, the delayed (20–24 h) E2 response provides an "*in vivo*" bioassay of the activation of the pituitary-ovarian axis. Serum E2 values of 50 pg/ml (range 40–80) or higher (increasing from a low baseline level) are consistent with CPP. Similarly, T values increasing to 100 ng/dl or higher upon GnRHa stimulation in boys are consistent with puberty, although data are limited due to the low incidence of CPP in males.

Lab evaluation in monitoring GnRHa treatment of central precocious puberty

The efficacy of treatment with GnRHa should be monitored by clinical, radiological and laboratory parameters, as discussed in various review articles (59, 113–115).

Laboratory evaluation should include ultrasensitive LH, FSH and sensitive and specific sex hormone levels (estradiol in girls, testosterone in boys). While clinicians may minimize or forgo blood tests if clinical indices of response to treatment are reassuring, in our center we use at least an initial laboratory assessment of treatment effectiveness approximately 3–5 months after initiation of GnRHa therapy, typically before the 3rd monthly depot-leuprolide injection, before the 2nd injection of a 12 week or 24-week depot preparation, or 2–3 months after placement of the histrelin implant. We suggest subsequent lab monitoring at least yearly, even if clinical evaluation is reassuring. Although the great majority of children respond to the recommended doses of GnRHa, occasional children show inadequate pubertal suppression and may benefit from an increase in the dosage of the GnRHa (for the depot Leuprolide preparations), more frequent administration (if allowed by insurance/healthcare regulations) or switching from an injectable form to a histrelin implant. Clinical trials for the available GnRHas report a range of response in each of the variables (116–122). The reader is referred to several reviews that are available on the different formulations of GnRHa available for treatment of CPP (59, 123–126).

There is disagreement regarding whether a baseline LH level is sufficient, or a GnRHa- stimulated level is preferable to monitor pubertal hormone suppression (117, 127). We have favored the GnRHa-stimulated values, traditionally "the gold standard" (128) which provide a more sensitive measure of LH suppression, although baseline values may be adequate, and "perfect" suppression of the pubertal hormones may not be necessary for a favorable outcome (129). A GnRHa-stimulated LH level <4 IU/L has been used to define biochemical suppression in most studies (59, 117, 126) although we and others (92) have observed lower values (typically <2.5 IU/L) in the great majority of children adequately responding to GnRHa therapy in our Center. As we have noted above, cutoff value variation in different studies can be related in part to the different immunometric LH assays employed. While we have used an LH value at 60 min after aqueous leuprolide stimulation for assessment of pubertal suppression for all patients in the past, we now use an LH measurement 60–90 min after Leuprolide-depot or triptorelin depot, except, of course, in children with the histrelin implant. The release of a large amount of rapidly absorbable GnRHa from the depot preparation has been shown to provide an intense stimulation of gonadotropins (92, 125).

With regard to random LH values, ultrasensitive LH < 0.6 IU/L has been proposed to define biochemical suppression but has not been rigorously studied (127). Others suggest an LH value <1 (130). Again, the cutoff value may be somewhat assay-dependent, which makes it difficult to compare different studies. It is important to note that random highly sensitive LH levels often fail to revert to a prepubertal range even when the pituitary-gonadal axis is fully suppressed (117, 131).

Estradiol and testosterone levels should be low in children adequately responding to GnRHa therapy, provided they are measured by appropriate sensitive and specific assays, as discussed above. In girls, estradiol levels should be <10 pg/ml and are, in fact, often undetectable even when measured by ultrasensitive assays (129). Testosterone values should be <10 ng/dl in pre-adrenarcheal boys, and <20–30 ng/dl in boys with less or more advanced adrenarche. That being said, occasional children (mostly boys in our experience) may have higher sex hormone levels and still show adequate pubertal suppression clinically and by LH monitoring. For this reason, and the fact that sex hormones are often measured by less sensitive and specific unextracted assays, measurement of sex steroids may have lower diagnostic sensitivity than measurement of LH levels.

Key Points: For laboratory monitoring of GnRHa therapy, measurement of GnRHa-stimulated LH (±FSH) levels is the "gold standard", with stimulated LH levels <2.5–4 IU/L indicating adequate suppression of the pituitary-gonadal axis. However, baseline LH < 0.6–1 IU/L may suffice to indicate acceptable suppression. Of note, LH values often remain above prepubertal values (0.3 IU/L) in children adequately treated with GnRHa. With good response to GnRHa therapy, serum E2 levels should be suppressed <10 pg/ml in girls, while in boys T levels <10–20 ng/dl are not always achieved and can still be compatible with adequate

suppression. Laboratory monitoring should always be used in conjunction with clinical and radiological evidence of response to therapy. This being said, machine learning algorithms to integrate multiple variables for the diagnosis of CPP are being developed (132).

Conclusion

Highly sensitive assays are essential to detect the early increase in LH levels at the beginning of puberty. The GnRHa stimulation test activates the entire pituitary-gonadal axis, thus representing a true *in vivo* "bioassay" that quantitates the ability of the child to synthesize sex hormones, the effective markers of pubertal effects on the body. This is particularly relevant for the diagnosis of CPP in girls, as a substantial percentage of them may not achieve a" pubertal" LH (usually considered >5 IU/L, but set at different cutoff values in different studies, as discussed above). Those girls with *bona fide* CPP who do not achieve a "pubertal" LH value on GnRHa stimulation, will most often achieve an E2 peak >50 pg/ml at 20–24 h, indicative of pubertal activation of the pituitary gonadal axis. In boys, measurement of the 20–24 h T response is not as crucial, as most boys with clinical signs of CPP will have stimulated LH responses >5 IU/L, however it may be helpful in corroborating the diagnosis of CPP in atypical cases. Treatment decisions need to be individualized and no one variable alone predicts adequate treatment response.

Data availability statement

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

Author contributions

KB: Writing – review & editing, Writing – original draft. LG: Writing – review & editing, Writing – original draft.

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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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Publisher's note

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Increasing prevalence of thyroid autoimmunity in childhood type 1 diabetes in the pre-COVID but not during the COVID era

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Introduction: Studies assessing longitudinal changes in the prevalence of autoimmune thyroiditis (AIT) among the pediatric population are limited. During the COVID-19 era, several papers proposed a rise in AIT cases. Our study aimed to analyze the prevalence of thyroid autoimmunity (TA) over a 10-year period spanning pre-pandemic and pandemic years in a population who are regularly screened for thyroid disturbances.

Materials and methods: This single-center retrospective cohort study analyzed data from 1,361 children and young adults with type 1 diabetes (T1D) treated between 2013 and 2022 in Hungary's largest pediatric endocrinology center. Results of anti-thyroid autoantibodies (anti-thyroid peroxidase/ATPO/and antithyroglobulin/ATG/), thyroid function tests (TFTs) and thyroid ultrasound examinations were obtained. Annual prevalence rates of TA and ultrasound-proven thyroiditis were calculated. Mean (± SD) follow-up period was 4.7 (± 2.8) years.

Results: The overall prevalence of TA among our T1D children was 22.8% ([20.3;25.5], 310 cases) with significantly more girls affected ($p<0.001$). From 2013 to 2022, TA prevalence rose from 15.9% to 20.6% ($p=0.041$). The increase was detected during the pre-pandemic years but not in the COVID-19 era. Ultrasound-confirmed thyroiditis was present in 80.0% of examined TA cases. Ultrasound positivity rate was stable during the study period. Among our children with TA, 28.5% exhibited clinically relevant thyroid-stimulating hormone (TSH) abnormalities (most commonly subclinical hypothyroidism) and/or were prescribed thyroid medication. Children with AIT had a significantly elevated risk of thyroid dysfunction compared to those with only thyroid autoantibody positivity ($p<0.001$).

Conclusion: Our results show a rise in the prevalence of thyroid autoimmunity among T1D children over the past decade, but our data do not support the assumed role of SARS-CoV-2 in the development of the disease.

KEYWORDS

type 1 diabetes mellitus, COVID-19, epidemiology, Hashimoto disease, pediatric endocrinology, post-acute COVID-19 syndrome, SARS-CoV-2, autoimmune thyroiditis

Introduction

During the coronavirus disease 2019 (COVID-19) pandemic era, numerous studies explored a potential connection between the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and different autoimmune diseases. The majority of large clinical studies and meta-analyses reported an increase in the incidence or an acceleration in the progression of type 1 diabetes (T1D) during the pandemic and particularly, following SARS-CoV-2 infection (1–4). However, some studies presented opposing results or found that the rise during the pandemic was within the range of the expected incidence (5–7). Similarly, while several studies indicated a rise in thyroid autoimmunity (TA, presence of anti-thyroid autoantibodies) and autoimmune thyroiditis (AIT, positive autoantibodies with inflammation of the thyroid gland as proven by ultrasound) during the pandemic, evidence remains controversial (8–12). Two studies specifically investigated the prevalence of TA in newly diagnosed T1D children, however, they also reported contradictory findings (13, 14). Our research group has also examined the prevalence of thyroid disturbances among the pediatric population post-COVID-19 but we - like many other scientists - faced a major limitation: the scarcity of pre-pandemic control data (15).

Type 1 diabetes (T1D) is the most common childhood endocrine disease. It is well-established that individuals with T1D have an elevated risk of developing other autoimmune conditions, such as AIT. According to the literature, the prevalence of AIT in children with T1D ranges from 17 to 30%, highlighting the strong association between these two autoimmune disorders (16).

As pediatric patients with T1D are at higher risk of developing AIT [most commonly Hashimoto's disease (17)], they undergo regular screening for TA based on the recommendation of the International Society for Pediatric and Adolescent Diabetes' [ISPAD, most recently updated in 2022 (18)]. To better understand the impact of the COVID-19 pandemic, we aimed to collect and analyze longitudinal data on AIT and TA in our pediatric T1D population.

The primary purpose of our study was to determine the annual prevalence of TA and ultrasound-proven thyroiditis among pediatric patients with T1D treated in one of Central Europe's largest pediatric endocrinology-diabetology centers over a 10-year period, covering both pre-pandemic (2013-2020) and pandemic (2021-2022) years. Additionally, we aimed to assess longitudinal trends in anti-thyroid peroxidase (ATPO) and antithyroglobulin (ATG) antibody titers along with thyroid function test (TFTs) results and data on thyroid medication during this period.

Materials and methods

Study setting, data collection and extraction

Our single-center retrospective cohort study was conducted at the Bókay Unit of the Pediatric Center, Semmelweis University, Budapest, Hungary. This center is responsible for the care of approximately 25% of all T1D children and adolescents in the country. Before exclusion, we obtained all patients' data with diabetes mellitus (DM) who received insulin treatment and had at least one visit between 1st of January, 2013 and 31st of December, 2022 at the inpatient and/or outpatient Endocrinology and Diabetes Unit of our Center. Clinical data and laboratory results were obtained from Semmelweis University's e-MedSolution software. Medical records were retrieved based on the BNO classification system, the Hungarian adaptation of the ICD-10 (The International Statistical Classification of Diseases and Related Health Problems, 10th Revision) classification released by the World Health Organization (https://icd.who.int/browse10/2019/en, accessed: 09.10.2024).

Data was completed and verified by four medical doctors and two medical students in agreement with our pre-specified research protocol. All arising questions were discussed in detail within the data extraction team.

Our study was conducted in accordance with the World Medical Association's Declaration of Helsinki. Due to the retrospective nature of our study, ethical approval was not required.

Inclusion and exclusion criteria

Inclusion criteria: all patients between 0 to 21 years of age (hereafter referred to collectively as "children"), who had been treated with T1D at our center between 2013 and 2022, and had at least one simultaneous ATPO and ATG measurements during

our study period. For the purposes of this study, we have chosen 21 years as the upper age limit, consistently with our clinical practice, where care is provided to individuals up to 21 years of age. Data on TA were obtained from both newly diagnosed and follow-up cases.

Exclusion criteria: patients over 21 years of age, children with diabetes forms other than T1D, and those who never underwent TA screening throughout the duration of our study.

Measured parameters and assay methodology

We collected the following parameters: clinical data of children (sex, date of birth, T1D diagnosis date), visit dates, thyroid autoantibody (ATPO, ATG) and TFTs (thyroid-stimulating hormone/TSH/, free thyroxine/fT4/) results from all years when the patient was screened as well as thyroid ultrasound findings for antibody-positive patients. Mean ages and time since T1D diagnosis were calculated according to the mid-point of each calendar year (1st of July). Laboratory analysis were carried out at the Immunology Laboratory of Semmelweis University with assay methodology and normal ranges shown in Table 1. If a child had more than one laboratory examination of a parameter in a calendar year, we used the last data for our analysis (or the last one before they started taking thyroid medicines in case of TSH and fT4). TSH levels above 6 mU/L or below 0.1 mU/L, and/or results that were consistently abnormal in a patient, were considered clinically relevant. TA was defined by at least one positive ATPO and/or ATG laboratory result obtained during our study period. Assay methodology and ranges of normal values of autoantibodies had changed multiple times over the examined period, therefore we calculated both absolute and relative values. Thyroid ultrasounds were deemed positive for thyroiditis if they showed enlargement and/or inhomogeneity and/or hyperemia in the thyroid gland. AIT was diagnosed if at least one autoantibody was positive and thyroiditis was confirmed by ultrasound examination.

Assessment of autoantibody results

Screening frequency of children was primarily based on ISPAD's guidelines (18), therefore patients without thyroid comorbidity did not go through autoantibody measurements each year. However, if a patient had presented a positive thyroid autoantibody result, they had been examined in all years afterwards. Due to the different screening frequencies between positive and negative children, prevalence data would be unrealistically high if not adjusted. Thus, in years when no laboratory examinations were carried out, we used the following protocol:

If the antibody titers were negative in both the preceding and subsequent years', we considered the intermediate year to be negative as well. When both were positive, we recognized it as positive. If they differed, the intermediate year was administered as missing data.

Description of the pre-pandemic and pandemic periods

We defined the pre-pandemic period as 2013 to 2020 and the pandemic period as 2021 and 2022. In the year of 2020, we did not expect a significant impact of the pandemic on our children, as a large Hungarian national representative study indicated that there were very few pediatric COVID-19 cases during the first pandemic wave (19).

TABLE 1 Laboratory assay methodology and ranges of normal values.

Laboratory parameter	Test used during the study period	Assay methodology, manufacturer	Units of measurement	Lower limit	Upper limit
Anti-thyroid peroxidase (ATPO)	1st of January, 2013 - 24th of March, 2014	ELISA, Aesku	UI/mL	–	40
	25th of March, 2014 - 20th of January, 2017	ECLIA, Roche	U/mL	–	63
	21st of January, 2017 - 11th of January, 2022	CLIA, Abbott	U/mL	–	5.6
	12th of January, 2022 - 31st of December, 2022	ECLIA, Roche	U/mL	9	34
Antithyroglobulin (ATG)	1st of January, 2013 - 24th of March, 2014	ELISA, Aesku	UI/mL	–	120
	25th of March, 2014 - 31st of December, 2022	ECLIA, Roche	UI/mL	–	115
Thyroid-stimulating hormone (TSH)	1st of January, 2013 - 31st of December, 2022	CLIA, Siemens	mU/L	0.35	4.94
Free thyroxine (fT4)	1st of January, 2013 - 31st of December, 2022	CLIA, Siemens	pmol/L	9	23.2

CLIA, Chemiluminescence Immunoassay; ECLIA, Electro-chemiluminescence Immunoassay; ELISA, Enzyme-linked Immunosorbent Assay.

Statistical analysis

Statistical analysis was carried out by a professional biostatistician. Each year, we calculated the prevalence using data from all children under care at that time. Consequently, the data for most children are included in our database for multiple years, corresponding to each year they were under care at our center. To calculate and compare prevalence data, and perform descriptive statistics, MedCalc Statistical Software version 22.023 was used (MedCalc Software bv, Ostend, Belgium; https://www.medcalc.org; 2020). Chi square comparison were conducted using StataCorp. (Stata Statistical Software: Release 18.5). The visualization was created using Microsoft Excel (Microsoft Corporation, 2016).

Results

Demographics

Between January 1, 2013 and December 31, 2022, a total of 1,667 patients with insulin-dependent DM were treated at our Clinic. We excluded 14 adults over 21 years old, 65 children who had another condition rather than T1D and 227 patients due to missing data and/or who were treated primarily at other centers. All in all, we included 1,361 T1D children's data in our final analysis. Mean (± SD) follow-up period was 4.7 (± 2.8) years. The girl-to-boy ratio was 637:724 (46.8% *vs* 53.2%). Detailed annual data are provided in Table 2.

Thyroid autoimmunity and ultrasound-confirmed thyroiditis

The overall prevalence of thyroid autoimmunity among our T1D children was found to be 22.8% ([20.3;25.5], 310 children). Girls were significantly more affected. (TA group: 106 boys, 204 girls, non-TA group: 618 boys, 433 girls; p<0.001, RR: 1.60 [1.43;1.78]).

From 2013 to 2022, the prevalence of TA increased from 15.9% to 20.6% (p=0.041). We observed this increase during the pre-pandemic years (2013 to 2019) but not in the COVID-19 era. Whilst analyzing the yearly prevalence differences, we found no significant difference between any consecutive years. Annual prevalence rates with confidence intervals (CI) are shown in Figure 1.

Of the children with TA who were screened with ultrasound at least once (n=260), 208 (80.0%) showed signs of thyroiditis on ultrasound, representing 67.1% of all children with TA. This resulted in an overall AIT prevalence of 15.3% in our study group. Among the 208 children with ultrasound-proven thyroiditis, one was later diagnosed with papillary thyroid carcinoma, as was one other child who did not show signs of thyroiditis. Although we performed an increasing number of ultrasounds throughout the years, the positivity rate remained stable. Yearly ATPO, ATG and ultrasound positivity rates can be seen in Table 3.

Given the multiple changes in assay methodology of ATPO and ATG titers during our examined period, we calculated both absolute and relative values in case of both antibodies. Detailed results of autoantibody titers are provided in Supplementary Tables 1, 2.

Clinically relevant thyroid dysfunction

We also analyzed the rate of thyroid hormone changes and data related to thyroid medication over this 10-year period in our children with TA. From the TFTs' analysis, we excluded children who did not have at least one TSH result during the study period (n=8). For children who were prescribed thyroid medication, we included only data preceding the initiation of treatment. Therefore, children who started their medication before 2013 (the beginning of our study period) were also excluded (n=20). Among the included 282 children, 44 (15.6%) exhibited clinically relevant elevation in TSH levels, while eight (2.8%) showed TSH reduction during the study period. Increased fT4 levels were observed in four cases, while decreased fT4 levels were noted in two children. The annual results are presented in Table 4. Detailed description of TSH and fT4 values can be seen in Supplementary Table 3.

Among the 310 children with TA, 74 (23.9%) were treated for hypo- and/or hyperthyroidism. Of these, 63 were initially prescribed levothyroxine to manage subclinical or clinical hypothyroidism, while two required levothyroxine following a total thyroidectomy due to thyroid carcinoma. Additionally, nine children were originally treated with antithyroid (thyrostatic) medications (thiamazole or propylthiouracil). During our study period, three children required treatment with both types of medication. Two of them began with

TABLE 2 Characteristics of included children.

	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Number of patients	483	513	520	631	680	732	689	738	745	713
Girls: boys (%)	48.2: 51.8	46.8: 53.2	48.5: 51.5	47.7: 52.3	46.9: 53.1	47.4: 52.6	45.9: 54.1	47.2: 52.8	45.9: 54.1	47.7: 52.3
Mean (SD) age (years)	12.1 (4.1)	12.3 (4.1)	12.5 (3.9)	12.3 (4.1)	12.4 (4.1)	12.4 (4.2)	12.4 (4.1)	12.4 (4.2)	12.5 (4.3)	12.6 (4.3)
Mean (SD) time from T1D diagnosis (years)	5.0 (3.8)	5.1 (3.9)	5.3 (3.9)	5.1 (3.9.)	5.2 (3.9)	5.3 (4.0)	5.3 (3.9)	5.4 (3.9)	5.6 (4.1)	5.7 (4.0)

T1D, type 1 diabetes.

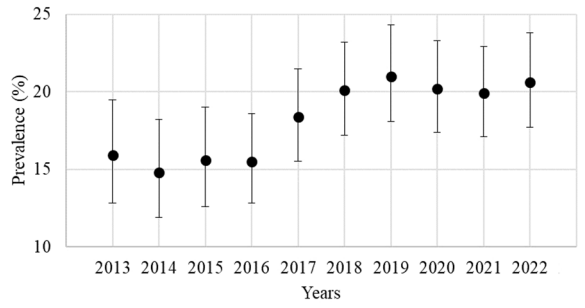

FIGURE 1
Annual prevalence rate of thyroid autoimmunity.

thiamazole and later transitioned to levothyroxine due to hypothyroidism, while one child started on levothyroxine and subsequently began taking thiamazole.

Altogether, out of the 302 children with TA who had at least one TSH result during the study period, 86 (28.5%) had clinically relevant TSH abnormalities and/or were prescribed thyroid medication.

The association between ultrasound findings and thyroid function results

We examined the connection between ultrasound positivity and thyroid function abnormalities among the children with at least one ultrasound and one TSH result (n=258). From this analysis, we excluded the two aforementioned children with papillary thyroid carcinoma, as they had started hormone replacement therapy following total thyroidectomy. The contingency table summarizing these data is presented as Table 5. Children with ultrasound-proven thyroiditis had a significantly elevated risk of having TSH abnormalities and/or being prescribed thyroid medication (p<0.001, RR: 6.24 [2.05;18.98]).

Discussion

In our retrospective cohort study involving 1,361 children and young adults with T1D, we found an increase in the prevalence of TA from 15.9% to 20.6% between 2013 and 2022. Interestingly, this increase was notable in the pre-COVID period (2013-2020) but not during the pandemic years (2021-2022). While the study's primary aim was to explore the potential effect of the COVID-19 pandemic on the prevalence of TA among T1D children, our results revealed a previously underreported trend: the rise in TA prior to the pandemic.

Numerous data shows that the prevalence rates of autoimmune diseases are increasing (20–22). A population-based cohort study of 22 million individuals in the UK found the largest incidence rate increase in coeliac disease, Sjogren's syndrome and Graves' disease, while Hashimoto's thyroiditis significantly decreased in incidence from 2000 to 2019 (23).

Studies involving the frequency of TA in pediatric T1D populations, mostly report on pooled prevalence data (17, 24). Cumulative incidence rate of TA was reported in children with T1D in a recent French study covering a similar study period like ours (2014 to 2020). They found an overall thyroid autoantibody frequency of 18% (25). The cumulative incidence of TA at 10 years of T1D was found to be 14% in a former study (26). However, longitudinal data on the trend of TA prevalence in the pediatric T1D population is scarce.

High prevalence of autoimmune thyroid diseases (AITDs) has been linked to various genetic, epigenetic, and environmental factors, though evidence on their specific effects remains inconsistent. Altered DNA methylation patterns, which regulate gene expression without altering the DNA sequence, may play a role in AITD development (27). Some studies have identified a relationship between vitamin D deficiency or insufficiency and AITDs (28, 29), while others found no such connection (30, 31). Additionally, insufficient selenium intake has been implicated in Hashimoto's disease occurrence (32). Higher iodine consumption has also been associated with an increased presence of anti-thyroid antibodies (33, 34). Stress-related disorders significantly elevated the risk of subsequent AITD, with a hazard ratio of 1.49 (1.42–1.56) observed in a large Swedish cohort study (35). However, the prospective Amsterdam AITD Cohort study found no link between stress exposure and *de novo* appearance of ATPO (36). Furthermore, an increasing number of studies are investigating the role of endocrine-disrupting chemicals (EDCs) in the onset and severity of AITDs, though research in this area remains limited (37). Among infectious agents, besides SARS-CoV-2, Epstein–Barr virus, Parvovirus B19, Human Herpesvirus 6A and Helicobacter pylori have all been suggested as contributors to AITD development (38–41).

TABLE 3 Thyroid autoimmunity results.

	2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
Prevalence of TA (%, ratio)	15.9 (77/483)	14.8 (76/513)	15.6 (81/520)	15.5 (98/631)	18.4 (125/680)	20.1 (147/732)	21.0 (145/689)	20.2 (149/738)	19.9 (148/744)	20.6 (147/713)
US positivity rate of children with TA (%, ratio)	81.8 (9/11)	82.8 (24/29)	85.4 (35/41)	82.0 (50/61)	88.8 (71/80)	79.8 (83/104)	75.2 (85/113)	79.4 (85/107)	83.1 (98/118)	83.5 (96/115)
Positivity rate of ATG (%, ratio)	3.8 (9/236)	9.8 (28/285)	14.0 (41/292)	12.0 (59/490)	12.0 (75/627)	12.6 (81/642)	11.5 (33/287)	6.7 (31/463)	11.3 (50/441)	9.6 (47/490)
Positivity rate of ATPO (%, ratio)	14.4 (35/243)	15.1 (46/304)	15.3 (52/340)	11.2 (58/518)	17.3 (102/591)	18.3 (118/644)	16.8 (66/392)	15.0 (76/505)	16.7 (74/443)	13.8 (68/493)

TA, thyroid autoimmunity; US, ultrasound; ATG, antithyroglobulin; ATPO, anti-thyroid peroxidase.

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TABLE 4 Annual results of thyroid function tests among children with thyroid autoimmunity.

		2013	2014	2015	2016	2017	2018	2019	2020	2021	2022
TSH results (%, number)	Low	0.0 (0)	0.0 (0)	3.3 (3)	1.6 (2)	0.0 (0)	0.6 (1)	0.0 (0)	0.8 (1)	0.8 (1)	0.8 (1)
	Normal	79.5 (31)	92.7 (38)	95.6 (86)	96.1 (122)	98.7 (152)	95.9 (162)	98.6 (138)	96.2 (126)	91.3 (116)	94.4 (119)
	High	20.5 (8)	7.3 (3)	1.1 (1)	2.4 (3)	1.3 (2)	3.6 (6)	1.4 (2)	3.1 (4)	7.9 (10)	4.8 (6)
	All	39	41	90	127	154	169	140	131	127	126
fT4 results (%, number)	Low	2.6 (1)	0.0 (0)	0.0 (0)	0.8 (1)	0.0 (0)	0.0 (0)	0.7 (1)	0.0 (0)	0.8 (1)	0.0 (0)
	Normal	97.4 (38)	100.0 (40)	100.0 (90)	99.2 (121)	100.0 (153)	100.0 (169)	99.3 (139)	99.2 (130)	99.2 (125)	99.2 (125)
	High	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)	0.8 (1)	0.0 (0)	0.8 (1)
	All	39	40	90	122	153	169	140	131	126	126

TSH, thyroid-stimulating hormone; fT4, free thyroxine.

Our hypothesis was that due to the high pediatric SARS-CoV-2 infection rate, we would observe an even higher TA prevalence among T1D children during the pandemic period. Our result do not support this initial hypothesis and it should be added, that even if we would have found an increasing prevalence during the pandemic period, our study design would only allow us to describe an association but not to state a cause-effect relationship. With the analysis of the pre-pandemic and pandemic era, we only had the opportunity to investigate the hypothesized change of the cumulative effect of numerous possible influencing factors (impact of SARS-CoV-2 and COVID-19 vaccines, delays in diagnosis making, changes in numerous habits such as eating, taking supplements, physical activity, decrease of other viral infections due to the restrictive measures and also other aspects which are not related to the pandemic and were discussed earlier).

To the best of our knowledge, only a few studies compared TA prevalence among T1D children between pre-pandemic and pandemic era with only one reporting annual data as well (14). Our results are in line with a small Turkish study, which found no significant difference in the frequency of concurrent thyroid autoantibodies (neither in ATPO nor in ATG) in newly diagnosed T1D children during the first pandemic year (February 2020 - January 2021) in comparison to the previous three years (February 2017 - January 2020) (13). In contrast, Al-Abdulrazzaq et al. published opposing results from Kuwait: children diagnosed with T1D during the COVID-19 pandemic (February 2020 - December 2022) had double the odds of testing positive for thyroid antibodies compared to those diagnosed earlier (January 2017 - February 2020). Additionally, according to their results, children with a positive history of COVID-19 were more likely to present with thyroid antibodies. In contrast to our finding of an increasing trend during the pre-pandemic years, they documented a stable prevalence between 2017 and 2019 (14).

Several studies reported an increased rate of TA during and after COVID-19 in adult, non-T1D populations. Increased prevalence of latent TA (ATPO positivity) was described in patients hospitalized for COVID-19 compared to healthy, pre-pandemic controls (8). Rossini et al. described a doubled prevalence of autoimmune thyroid disease (increased ATPO) in COVID-19 survivor adults compared to age and sex-matched controls (15.7% vs 7.7%) (9). An observational study from Spain documented a doubled diagnosis rate of Graves' disease in 2021, compared to data from 2017-2020 (10). In contrast, Lui et al. found only incidental TA positivity among adults recovering from COVID-19 (11). Moreover, a retrospective cohort study of pregnant women showed no significant difference in the incidence of new-onset thyroid dysfunction or autoimmunity between those who were SARS-CoV-2 seropositive and those who were seronegative postpartum (12). In a recent comprehensive review on the topic, the authors concluded that although SARS-CoV-2 can affect the thyroid gland and possibly cause subacute thyroiditis or TA, according to follow-up studies, survivors of COVID-19 showed no substantial long-term thyroid sequelae (42).

This issue is much less discussed in the pediatric literature than in adult studies. According to a recent narrative review focusing on the impact of the COVID-19 pandemic on thyroid diseases in the general pediatric population, there is currently no conclusive evidence linking SARS-CoV-2 with an increased incidence of TA in children. Additionally, they highlighted the paucity of data on the youth (43). A retrospective analysis by Shidid et al. observed no significant differences in the percentage of abnormal TSH results between the pre-pandemic (January 2017–October 2019) and pandemic periods (March 2020–October 2021). They focused on thyroid dysfunction without reporting on the frequency of TA (44). Consistently, a retrospective observational study from a tertiary

TABLE 5 Association between ultrasound positivity (thyroiditis) and thyroid function abnormalities.

	TSH Abnormalities/ Medication	No TSH Abnormalities/ Medication	Total number
Positive ultrasound result	76	131	207
Negative ultrasound result	3	48	51
Total number	79	179	258

TSH, thyroid-stimulating hormone.

pediatric endocrine center in the United Kingdom found no substantial changes in the presentation of thyroid dysfunction (hypo- and hyperthyroidism) in children before and after the pandemic. McCowan et al. also highlighted that the COVID-19 pandemic had no significant effect on fT4 or ATPO in hypothyroid patients (45). It is evident that there is a need for more pediatric-specific research.

Recent studies suggest that COVID-19-related thyroid dysfunction may occur through multiple mechanisms. SARS-CoV-2 can potentially disrupt thyroid function either by directly damaging thyroid cells via angiotensin-converting enzyme 2 (ACE-2) receptors or through indirect effects, such as triggering or exacerbating autoimmune processes (46–48). The virus may trigger autoimmune responses through various mechanisms, including molecular mimicry of SARS-CoV-2 proteins, bystander activation, epitope spreading during tissue damage, disruption of immunoprivileged barriers, polyclonal lymphocyte activation, and pathological activation of antigen-presenting cells via superantigens (48).

Our results support the findings of numerous previous large studies reporting female predominance among T1D children with TA (14, 17, 21, 24, 26). Additionally, older age and longer diabetes duration might increase the risk of developing TA (14, 24). In our study, the mean of these parameters remained stable during our study period so these factors did not bias our results.

Though the ISPAD protocol does not require ultrasound examinations for diagnosis and monitoring TA, we are proud that the number of performed ultrasound examinations grew remarkably during our study period and we could cover the vast majority of children among our TA population by 2022. It should be noted, that the positivity rate of ultrasound examinations was stable throughout the whole study period. We find this diagnostic tool inevitable in the early detection of malignancies.

Furthermore, we aimed to examine the differences between AIT and TA. While AIT specifically refers to an autoimmune condition in which the thyroid gland shows evidence of inflammation, TA is a broader term that encompasses the presence of thyroid-specific autoantibodies without necessarily having inflammation detectable by ultrasound. We found that children with inflammation of the thyroid gland (AIT) had a significantly greater likelihood of thyroid function abnormalities and subsequent thyroid medication use compared to those with only thyroid-specific autoantibodies and negative ultrasound results (TA). However, it is noteworthy that three children exhibited TSH alterations despite having no signs of thyroiditis on their ultrasound examinations.

Strengths

The main strength of our study is that it covers ten years of annual data on more than 1,300 children allowing us to assess the prevalence of TA longitudinally. The decade-long study period enables for a comprehensive assessment of trends in TA over time. By analyzing TA prevalence on a year-by-year basis, the study provides a more nuanced understanding of the trends compared to pooled data analysis. It has the ability to reflect on the increasing trend during a long-term period, while with the comparison of pre-pandemic and pandemic groups, one might assume that the difference between the pooled prevalences could be attributed to the impact of the pandemic. The inclusion of both pre-pandemic and pandemic years adds an important dimension to the understanding of the trends before and during the COVID-19 pandemic. Additionally, given that our center is responsible for the care of about 25% of all T1D children in Hungary and is one of the largest in Central Europe, we believe that our findings are representative of this region.

Limitations

As this was a retrospective study, we encountered instances of incomplete or missing data in certain cases. Possible reasons for these were missed annual check-ups by some patients, variations in protocols among specialists, overlooked screenings or insufficient blood sample volumes. Furthermore, the documentation regarding medication may have been inconsistent. Moreover, the laboratory test method used for the measurement of ATPO and ATG changed multiple times during our study period, thus we needed to present relative values of ATPO and ATG besides absolute ones. Unfortunately, as to our experiences, neither of them is ideal for precise comparison, nevertheless we indicated both results. Finally, it should be noted, that autoimmune diseases may develop months or years after an infection, so our study (ending in 2022) might not capture the long-term effects of the pandemic.

Conclusion

In our retrospective cohort study, we found an increase in the prevalence of thyroid autoimmunity among children with type 1 diabetes from 2013 to 2022. Unexpectedly, this growth was remarkable during the studied pre-pandemic period and it just stopped during the COVID-19 pandemic years. Therefore, our results among T1D children do not support the assumed role of SARS-CoV-2 in the development of thyroid autoimmunity.

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 requirement of ethical approval was waived by Prof. András Arató, Pediatric Center, Semmelweis University for the studies involving humans. Due to the retrospective nature of our study, ethical approval was not required. The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board also waived the requirement of written informed consent for participation from the participants or the participants'

legal guardians/next of kin because Due to the retrospective nature of our study and unidentifiable patients, written informed consent was not required.

Author contributions

VH: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EM: Conceptualization, Investigation, Methodology, Project administration, Writing – original draft, Writing – review & editing. DC: Data curation, Formal analysis, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing. LT: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. JT: Conceptualization, Data curation, Formal analysis, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RG: Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. FK: Data curation, Investigation, Project administration, Visualization, Writing – original draft, Writing – review & editing. AL: Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. AK: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. PT: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, 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.2024.1496155/full#supplementary-material

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Ultrasound and histopathological assessment of benign, borderline, and malignant thyroid tumors in pediatric patients: an illustrative review and literature overview

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Background: The risk of malignancy in thyroid nodules is higher in children than in adults, often necessitating a more aggressive endocrine and surgical approach. However, given that not all solid thyroid nodules are malignant, a more conservative approach may also be appropriate in certain cases.

Objective: This study aims to present an illustrative analysis of the pathological foundations underlying the sonographic appearance of benign, borderline, and malignant thyroid nodules in the pediatric population at a single tertiary thyroid center.

Methods : A total of 47 well-documented pediatric patients referred for thyroid surgery between 2010 and 2023 were analyzed. This retrospective assessment included an examination of demographic data, hormonal profiles, ultrasound findings, and histopathology reports.

Results: Ultrasound and histopathology of thyroid nodules provided insights into subgroup differentiation. Benign nodules like dyshormonogenetic goiter showed solid hypoechoic features on ultrasound and dense fibrosis on histopathology, while thyroid follicular nodular disease exhibited isoechoic nodules with halos, histologically revealing dilated follicles. In borderline tumors, well-differentiated tumor of uncertain malignant potential (WDT-UMP) nodules were hypo/hyperechoic with occasional capsular invasion, resembling papillary thyroid carcinoma (PTC) features histologically. Non-invasive follicular thyroid

neoplasm with papillary-like nuclear features (NIFTP) appeared as well-defined hypoechoic nodules with a hypoechoic rim, with histology showing follicular architecture and PTC nuclear features, but no invasion. Follicular tumor of uncertain malignant potential (FT-UMP) displayed hypo/hyperechoic patterns and indistinct borders, with uncertain capsular invasion and no PTC nuclear features. Malignant lesions showed distinct patterns: PTC as hypoechoic, irregular nodules with mixed vascularization, follicular thyroid carcinoma as large, hyperechoic nodules with invasive features, and poorly differentiated thyroid carcinoma (PDTC) as heterogeneous hypoechoic masses.

Conclusion: Because of the significant overlap in sonographic features among benign, borderline, and certain malignant thyroid lesions in pediatric patients, ultrasonography alone is insufficient for accurate risk stratification. This overlap necessitates referrals for fine-needle aspiration biopsy (FNAB) in children more frequently than in adults. Future studies utilizing artificial intelligence (AI) to predict clinical outcomes in thyroid nodule diagnostics may offer new advancements, particularly given the increasing number of pediatric patients with solid thyroid lesions.

KEYWORDS

NIFTP, WDT-UMP, FT-UMP, papillary thyroid carcinoma, follicular thyroid carcinoma

1 Introduction

Ultrasound (US) imaging plays a vital role in evaluating thyroid nodules, especially following the identification of low-risk (borderline) neoplasms, such as non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) and thyroid tumors of uncertain malignant potential: follicular tumor of uncertain malignant potential (FT-UMP) and well-differentiated tumor of uncertain malignant potential (WDT-UMP) (1, 2).

Epidemiological studies have shown a global increase in the prevalence of thyroid nodules, with rates among adults ranging from 33% to 68% (3, 4). In the general pediatric population, the incidence of thyroid nodules is lower (0.5% to 2%) compared to adults, though it is higher (3.5% to 31.5%) in children with autoimmune thyroiditis (AIT) (5–8). Pediatric thyroid nodules carry a 9.2%–50% risk of malignancy (ROM), compared to 5% to 15% in adults (9, 10). A 2023 study by Huang et al. noted an increasing incidence of thyroid cancer (TC) in individuals under 40 years of age in several countries, including Poland (11).

Given the higher malignancy risk in pediatric thyroid nodules compared to adults, treatment tends to be more aggressive (9, 10, 12, 13). However, not all solid thyroid nodules in children are malignant, suggesting that a more conservative approach could be appropriate in some cases (13). A prime example is the reclassification of encapsulated follicular variant of papillary thyroid carcinoma (EFVPTC) as NIFTP, which has significantly altered the therapeutic approach—from total thyroidectomy with radioiodine therapy to lobectomy with potential follow-up (14, 15).

For clinical pediatric endocrinologists, the updated 2022 World Health Organization (WHO) Classification of Thyroid Tumors has been particularly significant, highlighting the role of thyroid pathologists in decision-making (2). Most thyroid tumors originate from follicular epithelial cells and are categorized into benign, low-risk (borderline), and malignant neoplasms (2). Benign tumors include thyroid follicular nodular disease (TFND), follicular thyroid adenoma, follicular thyroid adenoma with papillary architecture, and oncocytic adenoma (OCA) of the thyroid (2). Low-risk neoplasms include NIFTP, FT-UMP, WDT-UMP, and hyalinizing trabecular tumor (2). Malignant neoplasms include follicular thyroid carcinoma (FTC), invasive EFVPTC, papillary thyroid carcinoma (PTC), oncocytic carcinoma of the thyroid, follicular-derived carcinomas, high-grade [poorly differentiated thyroid carcinoma (PDTC), differentiated high-grade thyroid carcinoma], and anaplastic follicular cell-derived thyroid carcinoma (2).

The presentation of TC, particularly PTC, in children is typically more severe than in adults, leading to more extensive surgical interventions, including total thyroidectomy, lymphadenectomy, and 131I therapy, which may result in significant long-term side effects (9, 10, 12, 13, 16). Therefore, ongoing research in the pediatric population should focus on improving the visualization of thyroid nodules and refining histopathological assessments to minimize the side effects of

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aggressive surgical approaches in children with benign and borderline tumors, who have a long life expectancy (2, 13, 16, 17).

Since the introduction of NIFTP in 2016 and the publication of the fifth edition of the WHO Classification of Thyroid Tumors in 2022, our center has revised pediatric histopathological assessments, leading to the diagnosis of 18 borderline tumors (2, 14, 15).

Sonographic assessment of rare borderline thyroid tumors in pediatric patients is not well defined. Therefore, we aimed to present an illustrative assay of the pathological foundations underlying the sonographic appearance of benign, borderline, and malignant thyroid nodules in the pediatric population at a single tertiary thyroid center.

2 Material and methods

2.1 Patients

For this illustrative study, we selected 35 well-documented cases that provided comprehensive data, including hormonal profiles, clinical information, and high-quality US images, which we could optimally match with high-resolution histopathological scans (Table 1; Figures 1–9). We also chose representative, high-quality US images from 12 patients, depicting all types of papillary carcinoma encountered in our thyroid center (Table 1; Figure 10). Our selection criteria were solely based on the best-documented, educational, and representative cases from our image collection.

This cohort of 47 patients was selected from the group of 262 pediatric patients (196 female patients; mean age of 13.1 years; age range, 6 to 18 years) who were referred for thyroid surgery to the University Children's Hospital in Krakow, a major tertiary pediatric center in Southeastern Poland, between 2010 and 2023.

2.2 Methods

The retrospective analysis of medical records involved evaluating thyroid function, as well as ultrasound and histopathological characteristics in patients with thyroid nodules. All hormonal and immune assessments were routinely conducted at the Department of Biochemistry, University Children's Hospital in Krakow, Poland. These assessments were performed on a single fasting blood sample, as previously described (18, 19). Thyroid-stimulating hormone (TSH) and free thyroxine (fT4) levels were measured using immunoassay methods with an ADVIA Centaur analyzer, while thyroid peroxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb) were assessed via radioimmunoassay using a Brams machine. All assessments were completed prior to the initiation of therapy, including levothyroxine or antithyroid drug treatment when required (except in patients with congenital hypothyroidism) and before any surgical intervention. Molecular analyses were routinely performed in cases of suspected genetic syndromes.

Thyroid ultrasonography (US) was conducted by certified pediatric endocrinologist and surgeon with significant experience in pediatric US (DJ > 20 years and AKW > 15 years). The examinations were performed using high-resolution systems: Voluson 730 GE Medical System (8–12 MHz linear-array

TABLE 1 Clinical evaluation of presented pediatric patients with thyroid nodules.

Patient	Sex	Age (years)	Clinical features of thyroid and/or risk group	TSH µIU/mL *N*: 0.3–4.0	fT4 pmol/L *N*: 10–25	TPOAb IU/ml *N* < 30	TgAb U/ml N<20	FNAB Bethesda score	Extent of surgery	Pathology
Figure 1										
A	F	18	Congenital hypothyreosis (CH) with goiter (two nodules in left lobe)	1.3	17.4	<30	<20	III/II (first FNAB) III/III (repeated FNAB)	L	Dyshormonogenetic goiter
B	F	8	Congenital hypothyreosis (CH) with goiter	1.7	16.8	<30	<20	III	L	Dyshormonogenetic goiter
Figure 2										
A	M	16	Goiter	3.5	0.9	1.5	0.1	III	L	TFND
B	M	14	Goiter, diabetes	1.5	15.6	<30	<20	IV	L	TFND
C	F	18	Goiter	1.6	15.6	<30	<20	III	L+I	TFND
D	F	18	Goiter	1.2	15.8	<30	588	IV	L	TFND

(*Continued*)

TABLE 1 Continued

Patient	Sex	Age (years)	Clinical features of thyroid and/or risk group	TSH µIU/mL *N*: 0.3–4.0	fT4 pmol/L *N*: 10–25	TPOAb IU/ml *N* < 30	TgAb U/ml N<20	FNAB Bethesda score	Extent of surgery	Pathology
Figure 2										
E	F	15	Goiter with a 5-cm nodule	1.7	16.3	<30	<20	III	L	TFND
Figure 3										
A	F	17	Goiter 68 mL	1.9	16	<30	<20	III	TT	TFND DICER+
B	M	12	Goiter 32 mL	1.2	17	<30	<20	III	TT	TFND DICER+
Figure 4										
A	F	16	Ultrasound evaluation	1.7	13	<0.8	<6.4	IV	L	Thyroid follicular adenoma
B	M	16	Ultrasound evaluation	4.2	12.9	<30	<20	III	L	Thyroid follicular adenoma
C	F	16	Goiter with hoarseness	1.3	14.2	<30	<20	II	L+I	Thyroid follicular adenoma
D	M	17 11/12	Goiter with hoarseness	1.2	15.4	<30	<20	IV	L+I	Thyroid follicular adenoma
Figure 5										
A	M	17	Goiter with a 20×17×23 mm nodule	2.12	15.3	<30	<20	III	L	Oncocytic adenoma
B	F	16	Brain radiotherapy (ALL), ultrasound surveillance	2.0	13.5	<30	<20	II, III, IV	TT	Oncocytic adenoma
C	M	17	Ultrasound evaluation	1.0	14.8	<30	<20	V	L	Oncocytic adenoma
D	M	16	Ultrasound evaluation	1.2	15.2	<30	<20	III	L	Oncocytic adenoma
Figure 6										
A	M	15	Ultrasound evaluation	5.2	16.6	<30	<20	III	L	NIFTP
B	M	12	Ultrasound evaluation	2.8	14.4	<30	<20	III	L	NIFTP
C	M	14	CH, dyshormonogenetic MNG	1.5	17.7	<30	<20	III	TT	NIFTP
Figure 7										
A	F	17.2	Ultrasound evaluation	1.5	16.6	34.2	20	III	TT	FT-UMP
B	F	17	AIT, ultrasound surveillance	1.5	11.6	30	1,217.8	III	L	FT-UMP
C	F	18	AIT, ultrasound surveillance	1.9	16.8	58	1,500	III	L	FT-UMP

(Continued)

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TABLE 1 Continued

Patient	Sex	Age (years)	Clinical features of thyroid and/or risk group	TSH µIU/mL *N*: 0.3–4.0	fT4 pmol/L *N*: 10–25	TPOAb IU/ml *N* < 30	TgAb U/ml N<20	FNAB Bethesda score	Extent of surgery	Pathology
Figure 7										
D	F	16	Ultrasound evaluation	1.3	15.5	<30	<20	III	L	FT-UMP
E	F	16	Brain radiotherapy (ALL), ultrasound surveillance	2.0	13.5	<30	<20	II, III, IV	TT	FT-UMP
F	M	16	Ultrasound evaluation	1.2	15.2	<30	<20	III	L	FT-UMP
Figure 8										
A	M	17	ALL, ultrasound surveillance	4.3	14.8	<30	<20	III	TT	WDT-UMP
B	M	16	2014 RTx total body, BMT (CGD), ultrasound surveillance	2.2	14.5	<30	<20	III	L+I	WDT-UMP
C	F	15	Goiter	2.3	1.04	1.7	0.1	I, II, III	L	WDT-UMP
D	F	17	Goiter with hoarseness	0.6	14.7	>600	>100	II	L	WDT-UMP
Figure 9										
A	F	15	Goiter	1.5	16.6	<30	<20	VI	TT	PTC
B	F	11	Goiter with hoarseness	1.5	14.5	<30	<20	III	TT	FTC
C	M	16	Goiter, 6 cm nodule	2.2	18	<30	<20	III	TT	Encapsulated FTC with angioinvasion
D	F	17	Goiter	1.46	15.3	<30	<20	VI	TT	PDTC
E	F	16	Goiter	1.4	13.8	<30	<20	VI	TT	PDTC
Figure 10										
A	F	17	US screening in thyroid clinic	1.4	15.7	>2,000	>1,000	VI	TT	PTC
A1	F	17	US screening in thyroid clinic	2.1	16.5	547.1	124.1	VI	TT	PTC
A2	F	10	US screening in thyroid clinic	1.2	12.9	143.4	93.4	VI	TT	PTC
B	M	10	US screening in thyroid clinic	2.6	14.5	<30	<20	VI	TT	PTC
B1	F	17	US screening in thyroid clinic	5.01	11.4	1,272.1	–	VI	TT	PTC
B2	M	16	US screening in thyroid clinic	2.3	14.7	<30	<20	VI	TT	PTC
C	F	12	US screening in thyroid clinic	<0.02	fT3 > 30.8 [*n*: 3.6–8.6]	<30	TgAb < 20 TRAb 4.0 IU/L [*n* < 1]	VI	TT	PTC

(Continued)

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TABLE 1 Continued

Patient	Sex	Age (years)	Clinical features of thyroid and/or risk group	TSH µIU/mL *N*: 0.3–4.0	fT4 pmol/L *N*: 10–25	TPOAb IU/ml *N* < 30	TgAb U/ml N<20	FNAB Bethesda score	Extent of surgery	Pathology
Figure 10										
C1	F	14	US screening in thyroid clinic	0.02	pmol/L fT4 70.1 fT3 13.4 [*n*: 3.6–8.6] pmol/L fT4 31.6	<30	TgAb < 20 TRAb 5.2 IU/L [*n* < 1]	VI	TT	PTC
C2	F	13	US screening in thyroid clinic	1.5	15.9	>1,000	>6,000	VI	TT	PTC
D	F	14	US screening in thyroid clinic	2.9	16.7	925.9	21.1	VI	TT	PTC
D1	F	8	US screening in thyroid clinic	0.9	17.1	748.2	22	VI	TT	PTC
D2	F	12	US screening in thyroid clinic	1.5	16.5	290.8	88.5	VI	TT	PTC

ALL, acute lymphocytic leukemia; CGD, chronic granulomatous disease; BMT, bone marrow transplantation; RTx, radiotherapy; AIT, autoimmune thyroiditis; CH, congenital hypothyreosis; MNG, multinodular goiter; L, lobectomy; L+I, lobectomy with isthmectomy; TT, total thyroidectomy; TFND, thyroid follicular nodular disease; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; WDT-UMP, well-differentiated tumor of unknown malignant potential; FT-UMP, follicular tumor of unknown malignant potential; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

transducer), Philips Epiq5 (L12-5 linear transducer), Philips iE22 (L11-3 linear transducer), and Samsung HS40 (LA3-16AD transducer), as previously described (18, 19). The analysis included ultrasound features of the thyroid gland based on the EU- TIRADS PL 2022 classification (Polish update of EU-TIRADS 2017) (20, 21) (Table 2).

Fine-needle aspiration biopsy (FNAB) results were categorized according to the 2023 (an update of 2017) Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) (22, 23). Surgical procedures included lobectomy, lobectomy with isthmectomy, or total thyroidectomy with central and, when necessary, lateral lymph node dissection (Table 1).

Histopathological evaluations were performed at the Department of Pathology, University Children's Hospital, and the Department of Pathomorphology, Jagiellonian University in Krakow, with MK serving as the responsible pathologist. Hematoxylin and eosin (HE)-stained tissue sections (deparaffinized, cut at 3.5 µm thickness) were scanned using the NanoZoomer SQ Hamamatsu at 400× magnification after routine diagnosis of thyroid nodules. Images were captured from the scans, with a scale bar positioned in the lower left corner.

Preoperative US images were analyzed with histopathology findings, with a focus on the nodule's shape, composition, echogenicity, margin characteristics, vascularity, extrathyroidal invasion, and presence of calcifications.

This study was approved by the relevant institutional review board (The Ethics Committee of the Jagiellonian University opinion number:118.0043.1.103.2024 issued on 19 April 2024). Written informed consent was obtained from all participants and/or their parents. 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.

3 Results

3.1 General overview

Between 2010 and 2023, 262 pediatric patients were referred for thyroid surgery at a major tertiary pediatric center. Histopathological diagnoses were updated according to the 2022 WHO Classification of Thyroid Tumors, revealing that 140 patients (53.4%) had benign nodules, 18 patients (6.9%) had borderline nodules, and 104 patients (39.7%) had malignant nodules (2). Among the 262 patients, the diagnoses were as follows: TFND in 84 (32.1%), thyroid follicular adenoma (TFA) in 25 (9.5%), OCA in 4 (1.5%), large thyroid cysts in 4 (1.5%), dyshormonogenetic goiter (DHG) in 2 (0.8%), therapy-resistant Graves' disease in 21 (8.0%), NIFTP in 3 (1.1%), WDT-UMP in 4 (1.5%), FT-UMP in 11 (4.2%), PTC in 89 (33.9%), invasive encapsulated follicular variant of PTC (IEFVPTC) in 1 (0.4%), FTC in 2 (0.8%), oncocytic carcinoma in 1 (0.4%), PDTC in 2 (0.8%), and medullary thyroid carcinoma (MTC) in 9 (3.4%).

A cancer predisposition syndrome was identified in four patients: one with Gardner syndrome and the columnar cell subtype of PTC, two with DICER1 syndrome and TFND, and one with Cowden syndrome and oncocytic carcinoma. For this illustrative review, we

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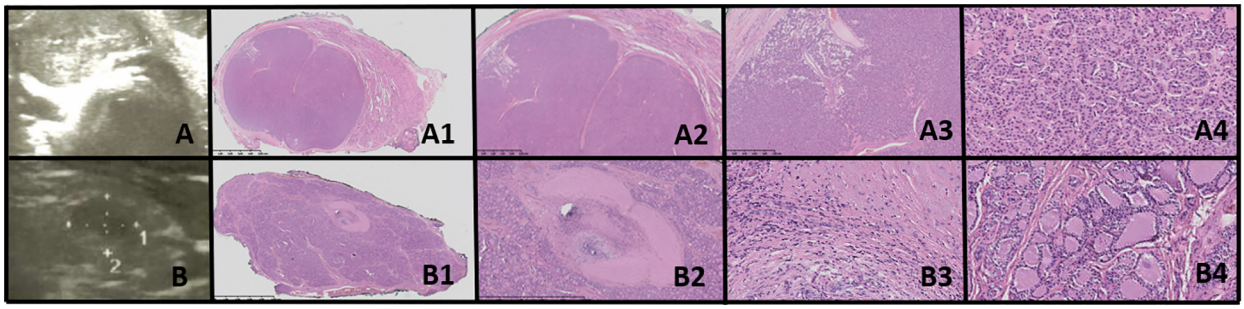

FIGURE 1
Dyshormonogenetic thyroid goiter (DHG). Columns represent: US and HE (magnification: A1, B1 ×5; A2, B2 ×50; A3, B3 ×1,000; and A4, B4 ×5,000). **(A)** Eighteen-year-old female patient; **(B)** 8-year-old female patient. US reveals a hypoechogenic nodule with well-defined borders and with hyperechogenic areas inside the nodule. In HE fibrosis, hemorrhages and inflammatory granulation tissue are seen. The structure is microfollicular and the nuclei are slightly enlarged and rarely overlap (A4, B3).

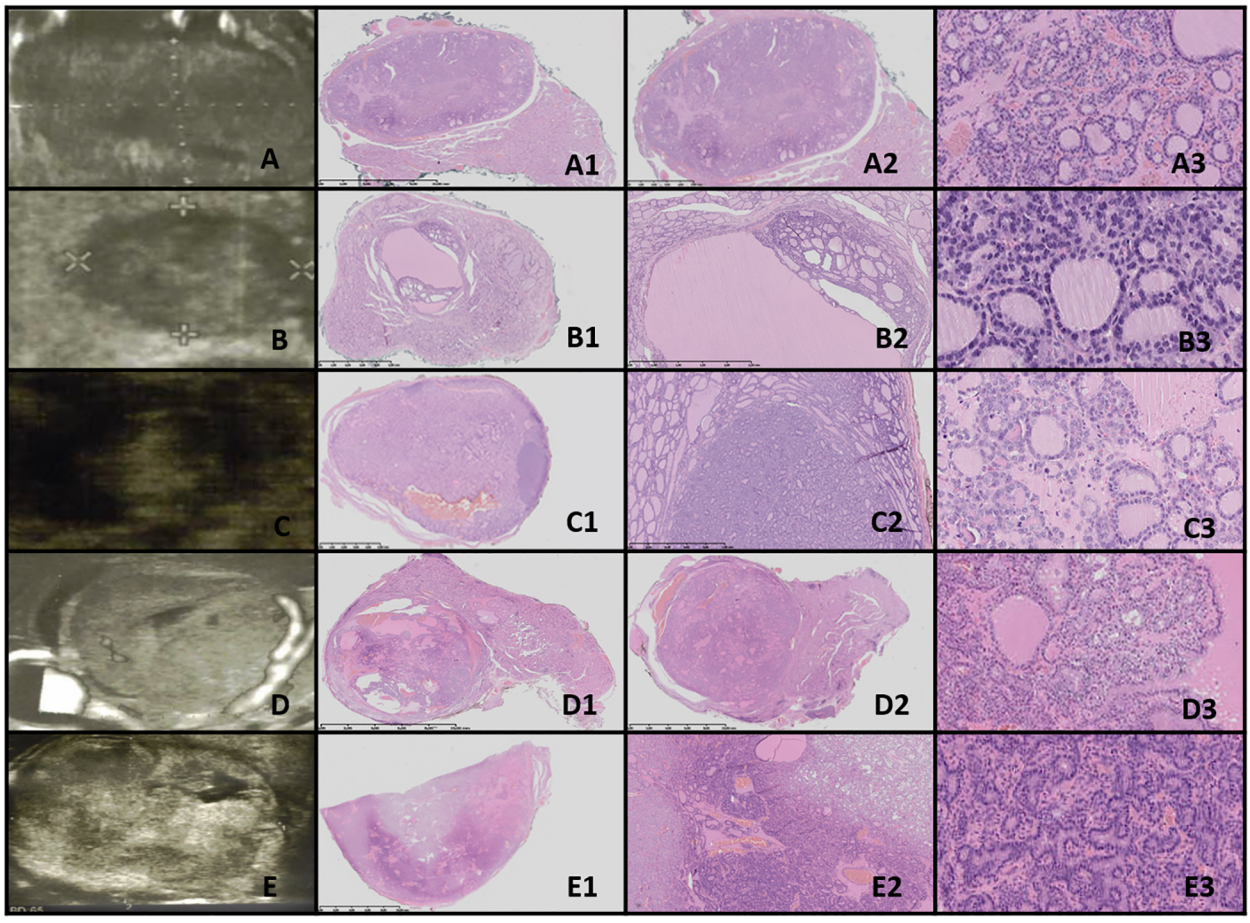

FIGURE 2
Thyroid follicular nodular disease (TFND). Columns represent US and HE (magnification A1–E1 ×5; A2–E2 ×50; and A3–E3 ×5,000). **(A)** Sixteen-year-old male patient with euthyroid goiter; **(B)** 14-year-old male patient with euthyroid goiter; **(C)** 18-year-old female patient with euthyroid goiter; **(D)** 18-year-old female patient with euthyroid goiter; **(E)** 15-year-old female patient with euthyroid goiter. In US, TFND is usually seen as a well-defined hyperechogenic nodule, surrounded by a hypoechogenic "halo" rim with mixed hypervascularity. In HE, macrofollicular (large follicles filled with pink colloid), medium-sized, and microfollicular structures are seen. Focally small fibrosis, hemorrhages, and papillary-like features are seen. The nuclei are a mixture of normotypical, slightly enlarged, and elongated, and they rarely have grooves. In patient **(E)**, ischemia (shrunk cells partially detached from the tissue matrix) in the central area of the nodule and clear-cell change are seen.

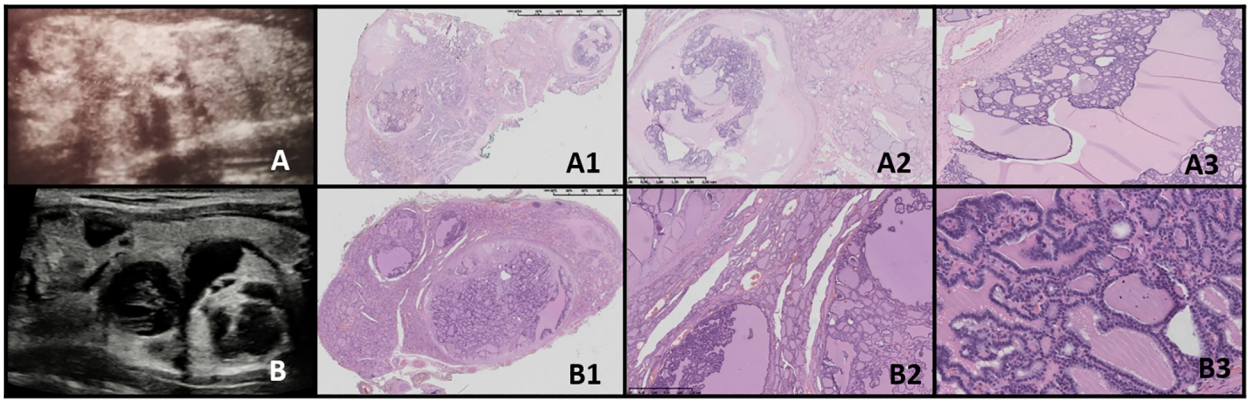

FIGURE 3
Thyroid follicular nodular disease (TFND) in patients with DICER1 syndrome. Columns represent US and HE (magnification: A1, B1 ×5; A2, B2 ×50; and A3, B3 ×5,000). **(A)** Seventeen-year-old female patient with euthyroid TFND; **(B)** 12-year-old male patient with euthyroid TFND. US shows multinodular goiter composed of hyper/isoechogenic solid-cystic nodules with macrocalcifications, especially in patient **(A)** In HE, the whole thyroid is built up by many hypocellular nodules filled with pink colloid. The hyperplastic nodules present a small, medium, and large vesicular structure and focally papillary arrangement (intrafollicular centripetal growth). Some of the nodules show areas of non-specific granulation, fibrosis, single calcifications, and a mixed-cellular inflammatory infiltrate with foamy macrophages containing hemosiderin. The remaining thyroid parenchyma is slightly congested.

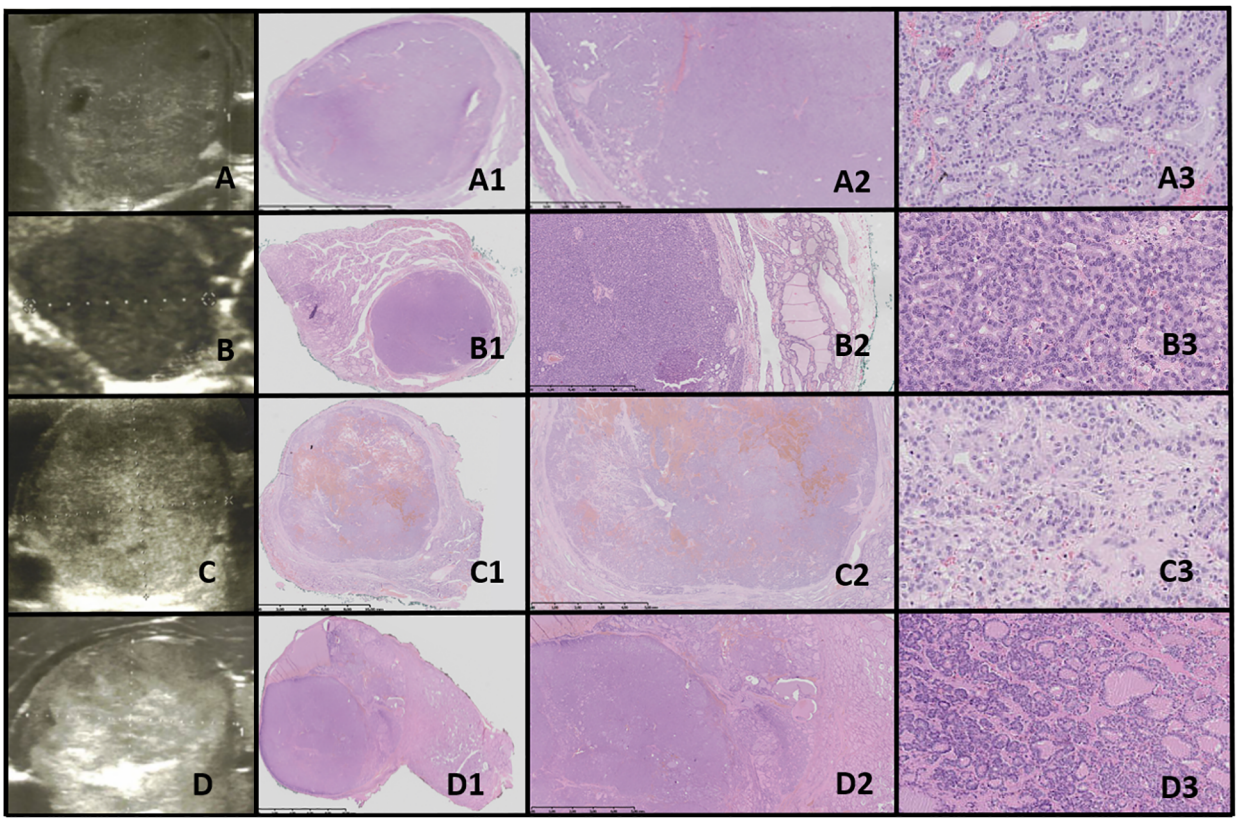

FIGURE 4
Thyroid follicular adenoma (TFA). Columns represent US and HE (magnification: A1–D1 ×5, A2–D2 ×50, and A3–D3 ×5,000). **(A)** Sixteen-year-old female patient with nodule found on US; **(B)** 16-year-old male patient with a nodule found on US; **(C)** 16-year-old female patient with euthyroid goiter with hoarseness; **(D)** 18-year-old male patient with euthyroid goiter with hoarseness. On US, a solitary, solid, round to oval, hypo/hyper/isoechogenic nodule is seen with well-defined hypoechogenic "halo" borders. On HE, the nodule is encapsulated, and the capsule is focally thickened and irregular. Pathological examination reveals no invasion through the capsule, the follicles inside a nodule are tightly packed, and the thyroid follicles adjacent to the nodule are constricted, larger (containing more colloid), but elongated. The nuclei are enlarged, with clearing and often overlap.

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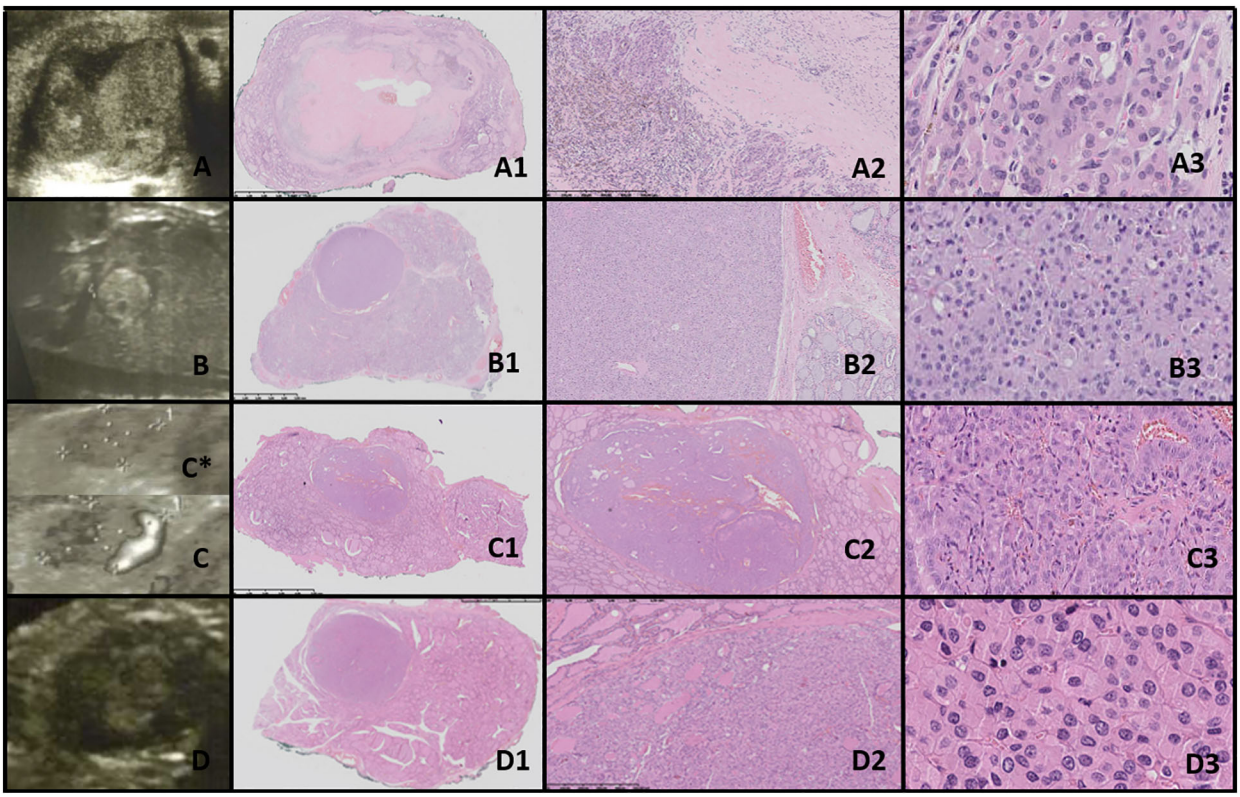

FIGURE 5
Oncocytic cell adenoma (OCA; Hürthle cell adenoma). Columns represent US and HE (magnification: A1–D1 ×5, A2–D2 ×50, and A3–D3 ×5,000). **(A)** Seventeen-year-old male patient with goiter; **(B)** 16-year-old female patient with a nodule found on US; **(C)** 17-year-old male patient with a nodule found on US; **(D)** 16-year-old male patient with a nodule found on US. US (**C, D** with power doppler, C*-without power doppler) reveals hyperechogenic nodules with small foci of hypoechogenic areas and increased mixed-type vascularity. HE examination reveals densely packed eosinophilic cells, and the hypoechogenic foci represent granular inflammatory tissue. Some of the cases might present with advanced fibrosis or contain medium-sized vessels (HE) and hyperperfusion in US (**A, C**, particularly). Cells are pleomorphic, with enlarged nuclei with prominent nucleoli.

selected 35 representative cases that covered the full spectrum of gray-scale US features observed in our clinic, correlating these with histopathological findings (see Figures 1–9; Tables 1, 3–5). Additionally, we included high-quality, illustrative US images from 12 patients that demonstrate the diverse sonographic presentations of PTC encountered at our center (Figure 10; Tables 1, 3).

The clinical and endocrine evaluations of 47 patients are summarized in Table 1, which also details the risk factors associated with the development of thyroid nodules, such as prior radiotherapy and chemotherapy for primary cancers.

3.2 Hormonal assessment

All patients with benign, borderline, and malignant tumors were euthyroid prior to surgery, with or without levothyroxine or antithyroid therapy as needed. AIT was confirmed in 15 patients before surgery (10 with PTC, 3 with FT-UMP, 1 with TFND, and 1 with WDT-UMP) (Table 1).

3.3 Risk factors

Notable risk factors included congenital hypothyroidism with goiter in three patients, brain radiotherapy for acute lymphoblastic leukemia (ALL) in one patient with OCA, brain radiotherapy for ALL in one patient with FT-UMP, chemotherapy for ALL in one patient with WDT-UMP, and total body irradiation prior to bone marrow transplantation for chronic granulomatous disease in one patient with WDT-UMP. Additionally, nodular AIT was diagnosed in 10 patients with PTC, 3 patients with FT-UMP, 1 patient with TFND, and 1 patient with WDT-UMP.

3.4 Ultrasound features

The ultrasonographic features of the subgroups are presented in Table 3 and in Figures 1–10. The evaluation included nodule composition, echogenicity, orientation, margin, calcifications, vascularization, gland and capsule shape, extrathyroidal invasion, and the presence of a “halo”. No distinct ultrasonographic patterns were identified to clearly differentiate benign, borderline, and malignant lesions. However, certain features were observed exclusively in malignant nodules, including microcalcifications, marked hypoechogenicity, lobulated or irregular ill-defined margins, and extrathyroidal invasion (Figures 9, 10; Table 3). Figure 10 displays various US images of pediatric PTC, highlighting different presentations: hypoechogenic nodules with

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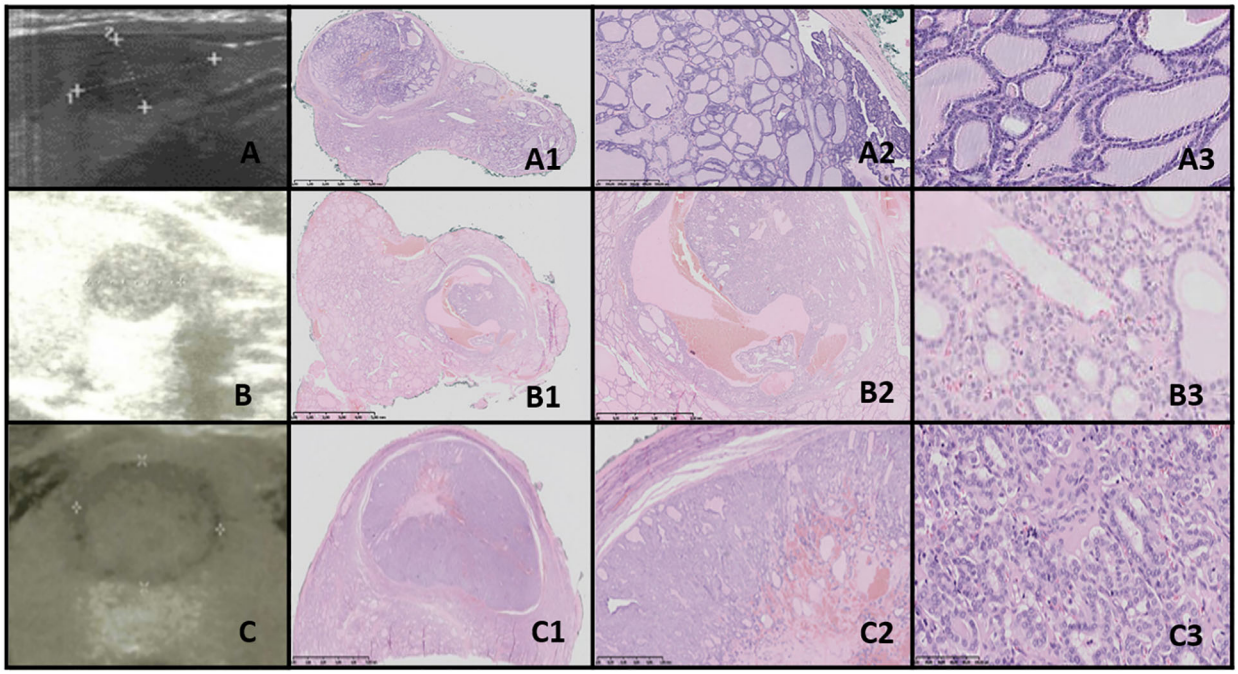

FIGURE 6
NIFTP. Columns represent US and HE (magnification: A1–C1 ×5, A2–C2 ×50, and A3–C3 ×5,000). **(A)** Fifteen-year-old male patient with a nodule found on US; **(B)** 12-year-old male patient with a nodule found on US; **(C)** 14-year-old male patient with a nodule found on US. US reveals a small, well-defined hypoechogenic nodule with acoustic (posterior) enhancement. HE reveals follicular structures, from micro- to macrofollicles; the nodule is round or oval, well-defined with or without fibrotic capsule, and there is an absence of capsular and vascular invasion. The nuclei have a set of PTC features (focally grade 3).

irregular or lobulated margins (A–A2), iso/hyperechogenic nodules with a halo (B–B2), nodules that are wider than taller (B1 and B2), diffuse sclerosing subtype (C–C2), and hypoechoic nodules with a surrounding hyperechogenic margin (D–D2). Apart from the C–C2 lesions, no microcalcifications were observed on US. As shown in image A, no increased vascularization was observed in small lesions.

3.5 Fine-needle aspiration biopsy

FNAB was performed in all nodules (Figures 1–10). The results of FNAB are presented in Table 1.

3.6 Surgical outcome

Total thyroidectomy was the initial approach for patients with a high suspicion of malignancy (Table 1; Figures 9, 10). Lobectomy, with or without isthmectomy, was more commonly selected as the first option for Bethesda category III cases and, less frequently, for category IV cases (Table 1). Surgical decisions were informed by FNAB results, as well as a comprehensive dataset that included patient history, age, gender, symptoms (e.g., large goiter, hoarseness), risk factors, US findings, and tumor growth potential, as previously described by Januś et al. (Table 1) (24).

3.7 Histopathological assessment

Histopathology remains the gold standard for differentiating and diagnosing thyroid lesions.

Table 4 and Figures 1–9 present the histopathological features of benign, borderline, and malignant lesions. The assessment included nuclear characteristics, cytoplasmic features, nodule margins, presence of necrosis, capsular and vascular invasion, tissue structure, cellularity, presence of calcifications, and characteristics of the surrounding thyroid parenchyma.

3.8 Ultrasound–histopathological general considerations

Table 5 presents the pathological basis of the US features observed in the study patients, including shape, margin, echogenicity, "halo" appearance, calcifications, and composition.

Thyroid nodule sections stained with HE were compared with corresponding thyroid US images. The colloid stained pink with eosin, while the nuclei of the follicular cells stained blue with hematoxylin, as previously described (1). Follicle size was inversely related to echogenicity: microfollicular nodules, with high nuclear density, appeared blue on HE sections and markedly hypoechoic on gray-scale US, whereas macrofollicular nodules, with low nuclear

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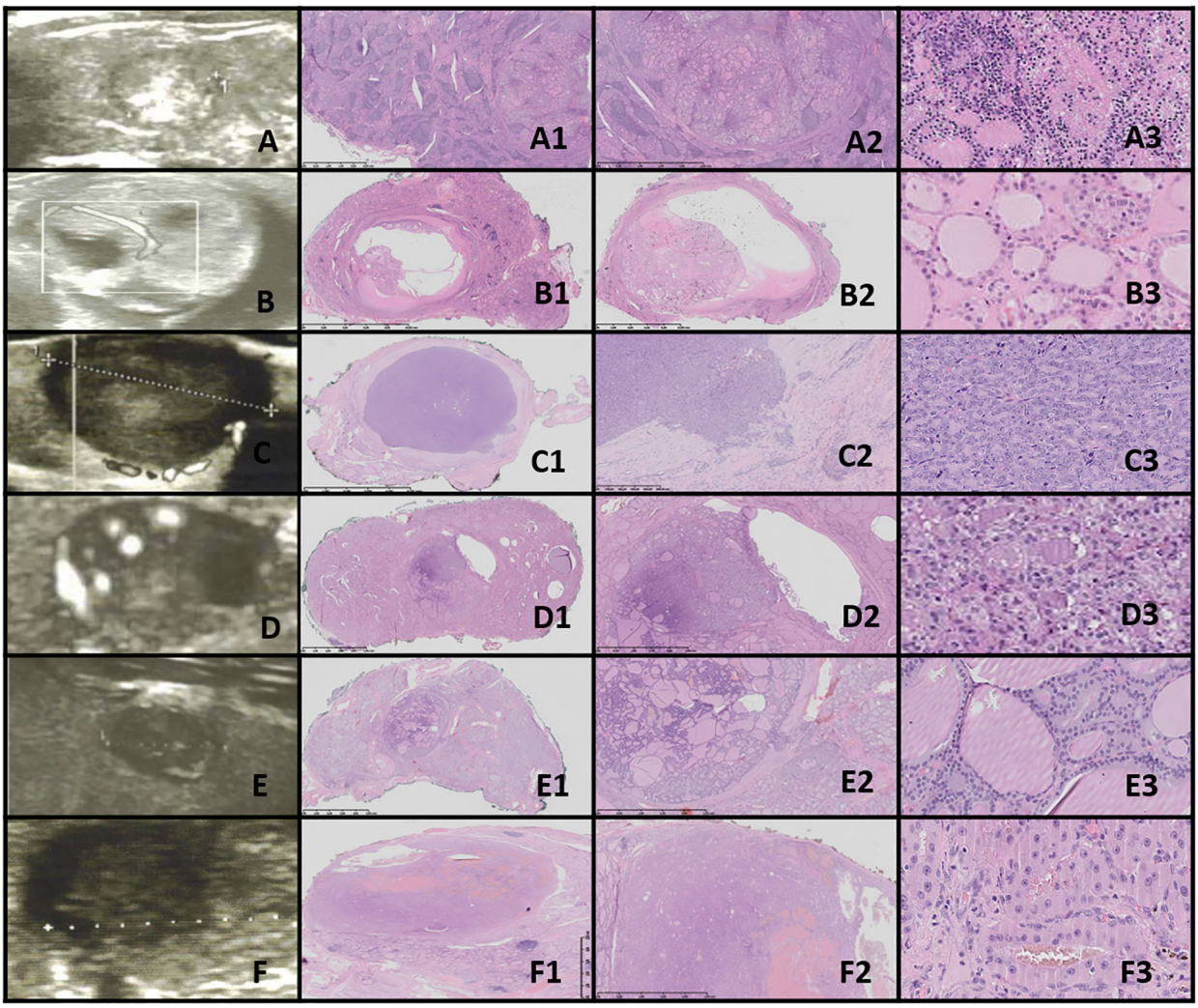

FIGURE 7
FT-UMP. Columns represent US and HE (magnification: A1–F1 ×5, A2–F2 ×50, and A3–F3 ×5,000). **(A)** Seventeen-year-old female patient; **(B)** 17-year-old female patient; **(C)** 18-year-old female patient; **(D)** 16-year-old female patient; **(E)** 16-year-old female patient; **(F)** 16-year-old male patient. In all patients, a nodule was found on US. US revealed small, foremost well-defined nodules with hypo- and hyperechogenic areas; however, focally the nodules' borders are hard to define. Vascularization is mixed in the nodules. HE reveals follicular structures, from micro- to macrofollicles, and the nodule is round or oval with uncertain foci of capsule invasion. There is absence of PTC nuclear features.

density, appeared pink on HE sections and were isoechoic or hyperechoic on US (1). The fibrous tissue capsule attenuated sound waves, presenting as a hypoechoic rim on US, particularly when the nodule had higher echogenicity (1). The margin characteristics of thyroid nodules observed in HE sections corresponded closely with the US findings, as reviewed by Yang et al. (1).

3.9 Ultrasound–histopathological evaluation within the subgroups

3.9.1 Benign thyroid nodules

3.9.1.1 Dyshormonogenetic goiter

In both of our patients with DHG, US imaging revealed an enlarged thyroid with solid hypoechoic nodules. Pathological examination in both cases revealed fibrosis, hemorrhage, and inflammatory granulation tissue. The thyroid architecture was microfollicular, with slightly enlarged and infrequently overlapping nuclei, confirming the diagnosis of DHG (Figure 1).

3.9.1.2 Thyroid follicular nodular disease (multinodular goiter)

Ultrasonographic evaluation revealed that TFND nodules were oval, isoechoic to hyperechoic, with a surrounding hypoechoic halo. Histological evaluation demonstrated variably sized dilated follicles with flattened to hyperplastic epithelium, with non-nodular thyroid tissue appearing reduced and compressed (Figure 2).

3.9.1.3 TFND in DICER1 syndrome

The US revealed multinodular goiter (MNG) composed of isoechogenic solid-cystic nodules with macrocalcifications, particularly notable in Patient A. The histopathology report

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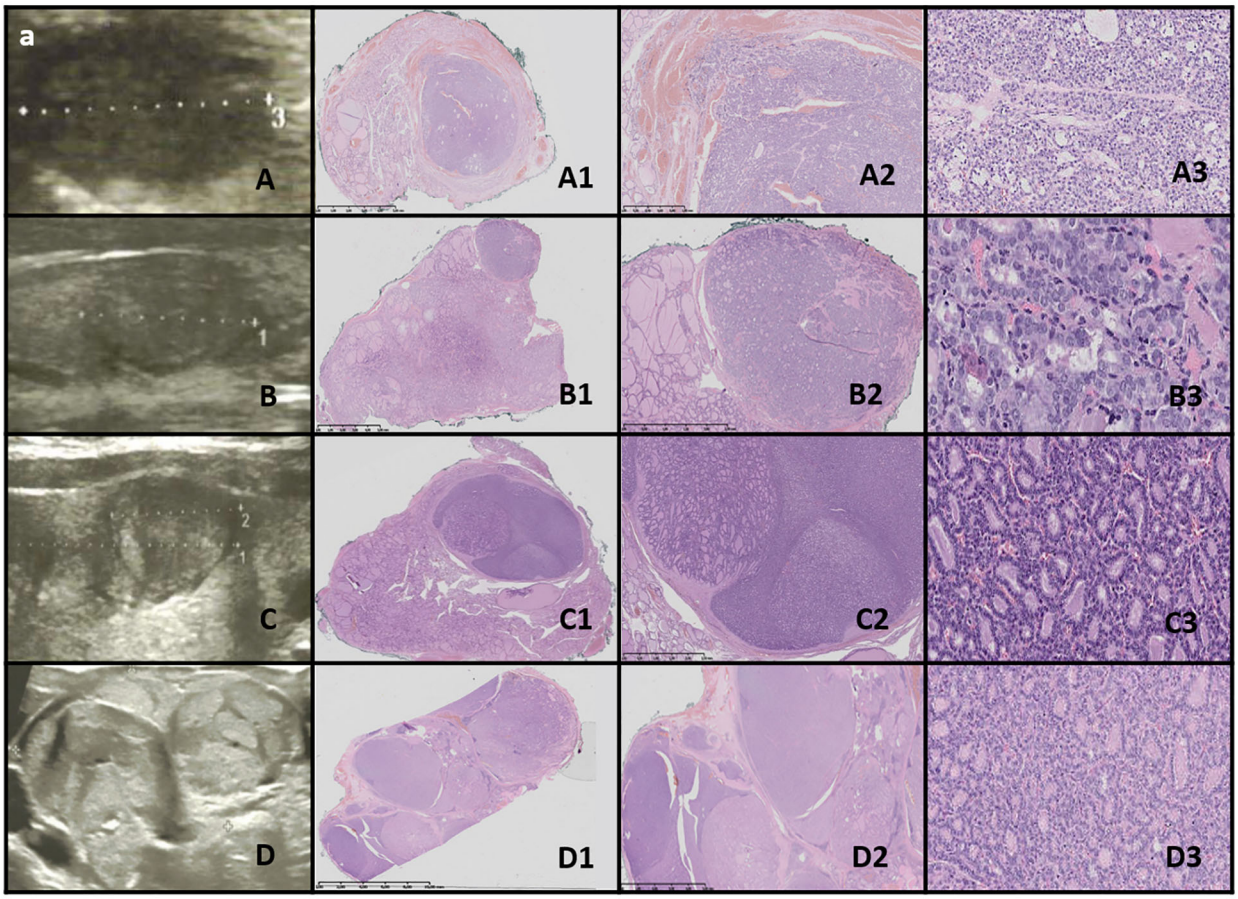

FIGURE 8
WDT-UMP. Columns represent US and HE (magnification: A1–D1 ×5, A2–D2 ×50, and A3–D3 ×5,000). **(A)** Seventeen-year-old male patient; **(B)** 16-year-old male patient; **(C)** 15-year-old female patient; **(D)** 17-year-old female patient. US reveals a medium-sized, quite well-defined hypoechogenic nodule in some cases with additional hyperechogenic areas. HE reveals follicular structures, predominantly microfollicles (hypoechogenic), and the nodule is oval, foremost well-defined but with the presence of capsular and vascular invasion, and the nuclei have PTC features.

revealed that the thyroid gland was composed of numerous hypocellular nodules containing pink colloid. The hyperplastic nodules exhibited a vesicular structure with focal areas of papillary arrangement, characterized by intrafollicular centripetal growth. Some nodules demonstrated areas of nonspecific granulation, fibrosis, isolated calcifications, and a mixed-cellular inflammatory infiltrate, including foamy macrophages containing hemosiderin. The remaining thyroid parenchyma was mildly congested (Figure 3).

3.9.1.4 Thyroid follicular adenoma

The US assessment revealed large, oval to round, solid nodules with mixed hypo-, hyper-, and isoechoic patterns, surrounded by a hypoechoic halo and displaying intranodular vascularization. Histological examination revealed encapsulated nodules with a capsule that was focally thickened and irregular, but without evidence of capsular invasion. The follicles within the nodules were tightly packed, while adjacent thyroid follicles were constricted, larger (containing more colloid), and elongated. The nuclei were enlarged, with nuclear clearing and frequent overlap (Figure 4).

3.9.1.5 Oncocytic adenoma

US revealed well-demarcated, round to oval hyperechogenic nodules with small hypoechogenic foci and increased mixed-type vascularity. Histopathological examination showed densely packed eosinophilic cells, with the hypoechogenic foci corresponding to granular inflammatory tissue. Some cases exhibited advanced fibrosis or medium-sized vessels, consistent with the hyperperfusion observed in the US (particularly in cases A and C). The cells were pleomorphic, with enlarged nuclei and prominent nucleoli (Figure 5).

3.9.2 Low risk/borderline tumors

3.9.2.1 NIFTP

The US of NIFTP revealed oval to round nodules with regular margins. The capsule appeared as a hypoechoic rim, except in markedly hypoechoic nodules. The echogenicity of NIFTP cases was generally hypoechoic, with an US artifact of acoustic (posterior) enhancement visible below the nodules (Figure 6).

Histopathological examination of NIFTP revealed follicular structures ranging from microfollicles to macrofollicles. The nodule was round or oval, well-defined, with or without a fibrotic capsule,

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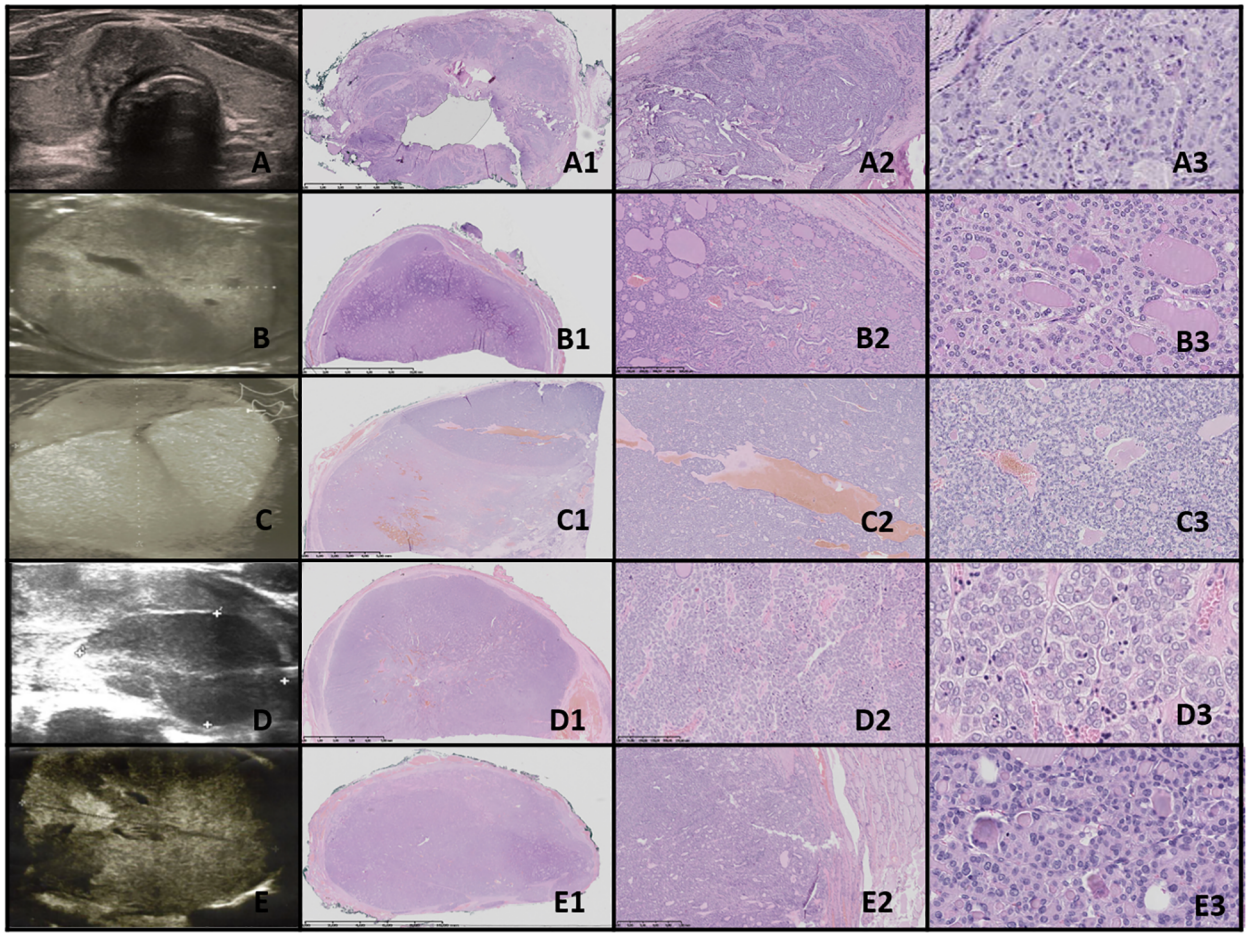

FIGURE 9
DTC and PDTC. Columns represent US and HE (magnification: A1–E1 ×5, A2–E2 ×50, and A3-E3 ×5,000). **(A)** Fifteen-year-old female patient with PTC; **(B)** 11-year-old female patient with FTC; **(C)** 16-year-old male patient with FTC; **(D)** 17-year-old female patient with PDTC; **(E)** 16-year-old female patient with PDTC. PTC **(A)**. US shows an irregular contoured, hypoechogenic nodule. In HE, the mixture of different-sized follicles built up from polymorphic cells with nuclei of "glassy" clearing and with grooves, invading through the capsule. FTC **(B, C)** and PDTC **(D, E)**. US shows large, hypo- and hyperechogenic nodules; however, although the outlines might seem to be well-defined, there are quite large areas of uncertain borders consisting of small hyperechogenic fragments. HE reveals follicular structures, from micro- to macrofollicles, which invade through the capsule and/or there is an angioinvasion **(D)**. The cells are pleomorphic and have large, overlapping nuclei. In PDTC, a set of neuroendocrine differentiation (salt-and-pepper nuclei) and trabecular structures can be found.

and without capsular or vascular invasion. The nuclei exhibited features of PTC, with a focal grade 3 nuclear score (Table 4).

3.9.2.2 FT-UMP

In our study group, all patients had a nodule incidentally detected on US. US imaging revealed a small, round to oval, predominantly well-defined nodule with hypo- and hyperechoic areas. In some cases, the borders of the nodules were difficult to delineate. Vascularization within the nodules was mixed (Figure 7).

Histopathological examination revealed follicular structures ranging from micro- to macrofollicles. The nodules were round to oval, with uncertain foci of capsular invasion. There were no nuclear features indicative of PTC (Table 4).

3.9.2.3 WDT-UMP

Ultrasound imaging revealed medium-sized, fairly well-defined hypoechoic nodules, sometimes with additional hyperechoic areas, appearing round to oval (Figure 8).

In one case, the nodule was found in the context of a multinodular goiter. Histopathological examination showed follicular structures, predominantly microfollicles. The nodule was oval and mostly well-defined, but with evidence of capsular and vascular invasion, and the nuclei displayed features of PTC (Table 4).

3.9.3 Malignant tumors

3.9.3.1 Papillary thyroid carcinoma

US imaging revealed an irregularly contoured, hypoechogenic nodule with increased mixed vascularization (central and peripheral) (Figure 9).

Histopathological examination revealed a mixture of follicles of varying sizes, composed of polymorphic cells with nuclei exhibiting "glassy" clearing and grooves, penetrating the capsule.

3.9.3.2 Follicular thyroid carcinoma

US imaging showed large hypo- and hyperechogenic nodules, though the borders, while appearing well-defined, often had areas of

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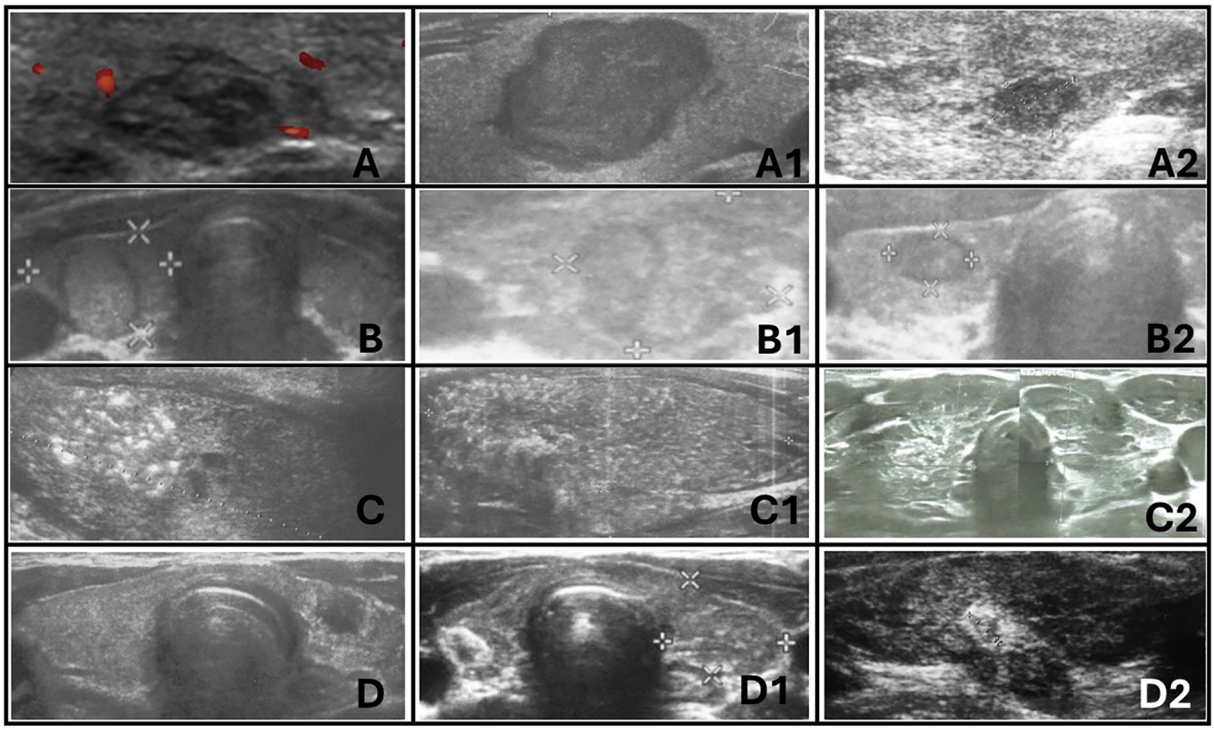

FIGURE 10
Ultrasonographic spectrum of papillary thyroid carcinoma in pediatric patients. **(A-A2)** hypoechogenic lesions with irregular margins; no increased vascularization in **(A)** and **(A2)**; no microcalcifications in **(A-A2)**; the shape is irregular oval, composition is solid. All patients with autoimmune thyroiditis (AIT). **(B-B2)** iso- and hypoechogenic lesions with `halo`; increased mixed vascularization and no microcalcifications in **(B-B2)**; the shape is taller than wider or wider than taller, composition is solid. Only **(B1)** with AIT. **(C-C2)** represents diffuse sclerosing variant of PTC. Extrathyroidal invasion is seen on **(C1)**. Vascularisation was increased in all lesions. All patients with AIT. **(D-D2)** represents hypoechogenic lesions surrounded by hyperechogenic irregular margin (histopathologically reported as fibrosis) in patients with autoimmune thyroiditis. No microcalcifications were seen but vascularization was increased in all lesions.

TABLE 2 EU-TIRADS-PL classification (20).

EU-TIRADS-PL category	Ultrasonographic features	Risk of malignancy	Indications for biopsy and/or further ultrasound monitoring
1	No nodules	Close to 0%	Ultrasound follow-up depending on clinical risk factors
2	Pure cysts Spongiform nodules	Close to 0%	FNAB not recommended (exception: therapeutic biopsy in symptomatic patients, e.g., cyst drainage); ultrasound follow-up depending on clinical risk factors
3	Normal/isoechoic or hyperechoic Ovoid or round shape Smooth margins No features of category 5	2%–4%	FNAB ≥ 20 mm
4	Hypoechoic Ovoid or round shape Smooth margins No features of category 5	6%–17%	FNAB ≥ 15 mm
5	Presence of at least one of the following features: • Marked hypoechogenicity • Irregular shape • Non-parallel orientation • Irregular margins • Microcalcifications • Extrathyroidal invasion*	>26%*	FNAB ≥ 5 mm*

Modified based on 2017 EU-TIRADS guidelines (21). *Modifications in comparison with the EU-TIRADS classification are marked: in EU -TIRADS 5: >10 mm FNAB, <10 mm consider FNAB or active surveillance; in EU-TIRADS 5: high risk 26-87%; in EU-TIRADS-PL 5- additional features: irregular shape and extrathyroidal invasion. Legend: FNAB — fine-needle aspiration biopsy.

TABLE 3 Ultrasonographic features of benign, borderline, and malignant lesions in the presented group of pediatric patients.

Ultrasound feature of the nodule		Benign					Borderline			Malignant
		DHG	TFND DICER–	TFND DICER+	TFA	OA	NIFTP	FT-UMP	WDT-UMP	DTC PDTC
Composition	Solid-cystic	–	+/–	++	–	–	+/–	+/–	–	–
	Solid	+	+	–	+	+	+	+	+	+
Echogenicity	Anechoic	–	–	+	–	–	–	–	–	–
	Hyper/isoechoic	–	+	+	+	+	–	+	–	+
	Mixed hypo/hyperechoic	+	+	+	+	+	+	+	+	+
	Hypoechoic	–	–	–	+	–	+	+	+	++
Orientation of the nodule	Parallel (wider than taller)	+	+	+	–	+	+	+	+	+
	Non-parallel (taller than wider)	–	–	++	+	+	+/–	–	+	++
Margin	Smooth	+	+	+	+	+	+	+	+	+
	Lobulated	–	–	–	–	–	–	–	–	+
	Irregular	–	–	–	–	–	–	–	–	+
	Ill-defined	+	–	–	–	–	–	–	–	+
Calcification	Macrocalcification > 1 mm	–	–	+	–	–	–	–	–	–
	Microcalcification <1 mm	–	–	–	–	–	–	–	–	+
Vascularization	Peripheral	+	+	+	+	–	+	–	–	+
	Mixed	–	+	+	+	+	+	+	+	+
	Central	–	–	+	+	–	+	+	+	+
Shaping of the gland and capsule	Present	+	+	+	+	+	+	–	+	+
Extrathyroidal invasion	Present	–	–	–	–	–	–	–	–	+
"Halo"	Present	+/–	+	+	+	+	+	+	+	+

DHG, dyshormonogenetic goiter; TFND, thyroid follicular nodular disease; TFA, thyroid follicular adenoma; OA, oncocytic adenoma; NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; FT-UMP, follicular tumor of uncertain malignant potential; WDT-UMP, well-differentiated tumor of uncertain malignant potential; DTC, differentiated thyroid carcinoma (PTC and FTC); PDTC-, poorly differentiated thyroid carcinoma.

uncertain demarcation with small hyperechogenic fragments (Figure 9).

Histopathology showed follicular structures ranging from micro- to macrofollicles with capsular and/or angio-invasion. The cells were pleomorphic, with large overlapping nuclei.

3.9.3.3 Poorly differentiated thyroid carcinoma

PDTC manifested as large, heterogeneous, hypoechoic masses on ultrasound (Figure 9).

Histopathological analysis revealed features suggestive of neuroendocrine differentiation, including salt-and-pepper nuclei and trabecular structures.

4 Discussion and literature overview

In this study, we presented the most common etiologies of thyroid nodules observed in our center, integrating US findings with corresponding histopathological features and referencing the current literature. The primary aim of this part was to provide a comprehensive overview of the latest knowledge on relatively newly identified borderline nodules, which are rare in pediatric patients, positioning them in relation to both benign and malignant thyroid tumors. By offering this comparative context, we aim to clarify the distinguishing characteristics of these borderline lesions and support their effective differentiation from other thyroid pathologies in clinical practice.

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TABLE 4 Histopathological features of benign, low-risk, and malignant lesions.

Histopathological features of the nodule		Benign					Borderline			Malignant
		DHG	TFND DICER–	TFND DICER+	TFA	OA	NIFTP	FT-UMP	WDT-UMP	DTC PDTC
Nuclei	Normotypical	++	++	––	+	–	––	–	––	–––
	Enlarged	+	+/–	+/–	+	+	+	+	+	+++
	Elongated	–	+	–	–	–	+	+	+	++
	Grooves	–	–	+/–	–/+	–/+	++	++	++	+++
	Clearing	–	–	+/–	+/–	+	++	++	++	+++
	Prominent nucleoli	–/+	–/+	+/–	–/+	++	–	–	–	–
Eosinophilic cytoplasm (intense pink)		+/–	–/+	+/–	+/–	+++	+/–	+/–	+/–	–/+
Well-defined and/or encapsulated nodule		+/–	+/–	+	+++	++	+++	++	++	–
Necrosis		–	–	–	–	–	–	–	–	+
Capsule invasion		–	–	–	–	–	–	+/–	+/–	+
Angioinvasion		–	–	–	–	–	–	+/–	+/–	++
Macrofollicular structure		++	++	++	+/–	+/–	+	+	+	–
Microfollicular structure		–/+	+/–	+	++	++	++	++	++	+
Papillary structure		–/+	–	–	–	–/+	–	–	–	++
Cellularity, nuclear crowding		–	–	+/–	+/–	+/–	+	+	++	+++
Atypia and polymorphism (of the benign and malignant, respectively)		–	–	+	–	–/+	+	+	+	++
Fibrosis		+/–	+/–	+	–	+	–	–/+	–/+	++
Hemorrhages/ischemia		+/–	+/–	+/–	+	++	–	–	–/+	–/+
Hyperplastic nodules		–	–	+	–	–	–	–	–	–
Intrafollicular centripetal growth		–	–	+	–	–	–	–	–	–
Macrocalcifications		+	+	+	++	++	–/+	–/+	–/+	+/–
Microcalcifications (Psammoma bodies)		–	–	–	–	–	–	–	–	+++
Congested parenchyma between the nodules		–	–	+	–	–	–	–	–	–

DHG, dyshormonogenetic goiter; TFND, thyroid follicular nodular disease; TFA, thyroid follicular adenoma; OA, oncocytic adenoma; NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; FT-UMP, follicular tumor of uncertain malignant potential; WDT-UMP, well-differentiated tumor of uncertain malignant potential; DTC, differentiated thyroid carcinoma (PTC&FTC); PDTC, poorly differentiated thyroid carcinoma.

4.1 Benign thyroid nodules

4.1.1 Dyshormonogenetic goiter

DHG is the second most common cause of congenital hypothyroidism, accounting for 10%–15% of all cases, following thyroid dysgenesis (25, 26). The incidence of DHG is approximately 1 in 30,000 to 50,000 live births, occurring twice as frequently in female patients (25, 26). Thyroid enlargement in DHG is due to defects in thyroid hormone synthesis (25, 26). Pathogenic variants in genes involved in this process include those responsible for thyroglobulin synthesis (TG), iodide transport across the basal (NIS/SLC5A5) and apical (PDS/SLC26A4) membranes of the follicular cell, hydrogen peroxide generation (DUOX2 and DUOXA2), iodide organification (TPO), coupling of mono- and diiodotyrosine (TPO), and the proteolytic breakdown of thyroglobulin and iodide recycling (IYD/DEHAL1) (25, 26). A deficiency in circulating thyroid hormones leads to the activation of TSH secretion, which, in turn, causes hyperplasia of the defective thyroid gland (27). This can result in the development of tumors, such as FTC and PTC, and, less commonly, follicular adenoma (27). To date, approximately 30 cases of DHG associated with thyroid carcinoma have been reported, including four pediatric cases involving a newborn and children aged 6, 14, and 17 years (26, 28–30).

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TABLE 5 Pathologic basis of ultrasound features (shape, margin, echogenicity, "halo", calcifications, and composition).

Feature	Sonography	Histopathology	Tumor type	Figures
Shape	Wider than taller, round	Horizontal growth	DHG TFND DICER– TFND DICER+ OA NIFTP FT-UMP WDT-UMP PTC	Figure 1 Figure 2 Figure 3 Figure 5 Figure 6 Figure 7 Figure 8 Figure 10B2
	Taller than wider	Vertical growth	TFND DICER+ TFA WDT-UMP PTC	Figure 3 Figure 4 Figure 8 Figure 10
Margin	Smooth	No invasion	DHG TFND DICER– TFND DICER+ TFA OA NIFTP FT-UMP WDT-UMP PTC	Figure 1 Figure 2 Figure 3 Figure 4 Figure 5 Figure 6 Figure 7 Figure 8 Figure 10B2
	Lobulated	Expansile growth, group of tumor cells "pushing" into surrounding follicles	PTC	Figure 9A Figure 10A1
	Irregular	Irregular	PTC	Figure 10C-C2 Figure 10D-D2
Echogenicity	Marked hypoechoic	Tightly packed microfollicles or solid nests of tumor cells	PDTC PTC	Figures 9D, E Figure 10A1, A2
	Hypoechoic	Small-to-medium follicles	DHG TFND DICER– TFA OA NIFTP FT-UMP WDT-UMP PTC FTC	Figure 1 Figure 2 Figures 4B, C Figure 5 Figure 6 Figure 7 Figure 8 Figure 9A Figures 9B, C
	Isoechoic to hyperechoic	Normal to macrofollicles	TFND DICER– TFND DICER+ TFA OA FT-UMP WDT-UMP FTC PTC	Figure 2 Figure 3 Figures 4A, D Figure 5 Figure 7 Figure 8 Figures 9B, C Figure 10B
Hypoechoic rim (halo)	Present	Capsule around normal-sized follicles or macrofollicles	DHG TFND DICER– TFND DICER+ TFA OA NIFTP FT-UMP WDT-UMP PTC	Figure 1 Figure 2 Figure 3 Figure 4 Figure 5 Figure 6 Figure 7 Figure 8 Figure 10B
	Absent	Tightly packed microfollicles	FT-UMP WDT-UMP	Figure 7 Figure 8
Calcifications	Micro	Psammomatous small calcifications	PTC	Figure 10C-C2
	Macro	Coarse calcifications	TFND DICER+	Figure 3A

(Continued)

TABLE 5 Continued

Feature	Sonography	Histopathology	Tumor type	Figures
Composition	Isoechoic nodule with cystic areas	Macrofollicular nodule with cystic areas	TFND DICER+	Figure 3
	Solid	Solid, microfollicular, dense papillary or trabecular	DHG TFND DICER– TFA OA NIFTP FT-UMP WDT-UMP PTC, FTC, PDTC	Figure 1 Figure 2 Figure 4 Figure 5 Figure 6 Figure 7 Figure 8 Figures 9, 10

DHG, dyshormonogenetic goiter; TFND, thyroid follicular nodular disease; TFA, thyroid follicular adenoma; OA, oncocytic adenoma; NIFTP, non-invasive follicular thyroid neoplasm with papillary-like nuclear features; FT-UMP, follicular tumor of uncertain malignant potential; WDT-UMP, well-differentiated tumor of uncertain malignant potential; PDTC, poorly differentiated thyroid carcinoma.
Modified according to Yang et al. (1).

In both of our patients with DHG, US imaging revealed an enlarged thyroid with solid hypoechoic nodules, consistent with previous reports (31). FNAB yielded results of V and III, respectively, indicating a ROM of approximately 28% (9, 12). As a result, uneventful lobectomies were performed at ages 8 and 18 years. The average age of surgery due to nodular goiter in DHG, as reported in the literature, is 16 years (32).

Histologically, DHG is characterized by markedly hypercellular nodules, with predominant patterns including solid, microfollicular, macrofollicular, trabecular, and insular nodules (26). Additional features include papillary hyperplasia, absence of colloid, frequent internodular bizarre cells, and bridging fibrosis, as reviewed by Bychkov et al. (26).

4.1.2 Thyroid follicular nodular disease (multinodular goiter)

The term "follicular nodular disease (FND)" was introduced in the WHO 2022 Classification to describe multifocal hyperplastic or neoplastic lesions occurring in the clinical context of MNG (2). TFND is the most common thyroid gland disorder, detected in 60% of benign tumors in this study. Ninety percent of affected patients are women (33). Autopsy reports estimate the prevalence of TFND at 10% to 40% (33). The ROM in TFND is between 3% and 5% (33, 34). Most patients with TFND are asymptomatic and euthyroid, as also observed in this study. Globally, iodine deficiency is the leading cause of TFND, while in Western countries, AIT is more prevalent (33). In the pediatric population, TFND more commonly develops during adolescence (33). The genetic basis of the disease, especially in pediatrics, includes tumor-predisposing syndromes such as familial adenomatous polyposis, PTEN hamartoma tumor syndrome (Cowden syndrome), Werner syndrome, Carney complex, Pendred syndrome, McCune–Albright syndrome, and DICER1 syndrome (9, 12, 13, 35–40).

As reviewed by Satturwar et al., TFND nodules can display a variety of US features, including isoechoic or hyperechoic nodules with a hypoechoic halo, a sponge-like or honeycomb pattern, anechoic areas containing colloid, and internal calcifications (33).

Satturwar et al. reported that TFND nodules may present a wide range of histological patterns, from colloid-rich and microfollicular to hypercellular and microfollicular (33). Secondary changes such as fresh or old hemorrhage, follicular rupture with a granulomatous response, fibrosis, calcification, and even osseous metaplasia may also be observed (33). Some cystically dilated follicles may exhibit papillary projections (Sanderson polsters) that mimic papillary carcinoma, although they lack the nuclear features characteristic of papillary carcinoma (33, 41–43).

4.1.3 TFND in DICER1 syndrome

The DICER1 gene, located on chromosome 14q32.13, plays an important role in normal thyroid gland development (44–47). Multiple thyroid abnormalities have been identified in DICER1 syndrome, in addition to other non-thyroidal neoplasms (44–47). In 2011, Rio et al. reported that individuals carrying a germline pathogenic variant of DICER1 have an increased predisposition to developing TFND/MNG, with a 16- to 24-fold higher risk of TC compared to the general population (46, 47). Somatic DICER1 pathogenic variants are associated with thyroblastoma and childhood-onset PDTC, whereas germline variants are linked to TFND, follicular adenoma with papillary architecture, PTC, and FTC (47).

In our study, US revealed MNG composed of isoechogenic solid-cystic nodules with macrocalcifications, particularly notable in Patient A as previously reported (46, 47).

As reviewed by Riascos et al., TFND DICER+ is histologically characterized by the presence of multiple bilateral nodules showing follicular proliferations (47). These nodules may present as adenomatous nodules, macrofollicular-pattern nodules, well-circumscribed adenomas, or nodules with intrafollicular centripetal papillary growth, similar to those observed in our study (47). This growth pattern is often referred to as papillary hyperplasia or papillary adenoma, but it lacks the nuclear features typical of PTC (48). In patients with multiple adenomatous nodules, Cowden syndrome should be excluded (49). The suspicion of DICER1-related pathogenesis should be heightened when variable involutional changes are observed in the non-nodular thyroid parenchyma, as noted in our study group (48).

4.1.4 Thyroid follicular adenoma

TFA is a benign, encapsulated tumor characterized by thyroid follicular cell differentiation, without capsular or vascular invasion, and lacking the nuclear features of PTC (50).

The incidence in the general population is approximately 3%–5%, predominantly affecting adults, typically in the fifth to sixth decades of life, with a higher prevalence in female patients (50, 51). The etiology is usually sporadic, though it may occur following radiation exposure or as a result of iodine deficiency (50, 52). TFA can also be observed in the pediatric population, particularly in association with familial tumor syndromes such as PTEN syndrome, Carney complex, MEN1 syndrome (Wermer syndrome), and McCune–Albright syndrome (50, 53, 54). Most patients are euthyroid, though hyperthyroidism can occur in cases of hyperfunctioning adenomas, especially in McCune–Albright syndrome (50).

According to Agarwal et al., US in TFA typically shows solid or solid-cystic nodules with smooth, well-defined margins, homogeneous or heterogeneous echotexture, isoechoic or hypoechoic characteristics, and sometimes a peripheral hypoechoic halo (50, 55–57). Blood flow is either absent or low (50, 55–57). In our study, US assessment revealed large, oval to round, solid nodules with mixed hypo-, hyper-, and isoechoic patterns, surrounded by a hypoechoic halo and displaying intranodular vascularization.

As reviewed by Agarwal et al., histopathological evaluation of TFAs shows that they are architecturally and cytologically distinct from the surrounding gland, causing compressive changes in the adjacent thyroid tissue (50). They are encapsulated by a thin to moderately thick capsule (50). The nodule structure can vary, presenting as normofollicular, microfollicular, macrofollicular, or solid/trabecular (50). A focal papillary pattern may be seen in hyperfunctioning adenomas and follicular adenomas with papillary hyperplasia (50). The nuclear features of PTC are absent (14, 50).

4.1.5 Oncocytic adenoma

Oncocytes are enlarged, polygonal to square-shaped epithelial cells with distinct cell borders and a voluminous, granular, eosinophilic cytoplasm, resulting from the accumulation of mitochondria (58). Oncocytic change can be observed in various benign conditions, such as AIT, TFND, or MNG, particularly in patients who have undergone head and neck radiotherapy, systemic chemotherapy, or in benign and malignant thyroid neoplasms (59).

Oncocytic tumors (OCTs), formerly known as Hürthle cell tumors, are rare follicular-derived thyroid neoplasms, accounting for less than 5% of all thyroid tumors (2, 60). OCTs can be classified as either adenomas or carcinomas (61). According to Bhattacharyya et al., OCTs are more common in women (68%) and typically occur in the sixth decade of life (62). While most OCTs are benign (OCA), up to 40% have been reported to be malignant [oncocytic cell carcinoma (OCC)] (61, 63).

To date, only three cases of OCA have been described in children (60, 61, 64). In our study, we presented four pediatric cases, including one patient who had received brain radiotherapy for acute lymphocytic leukemia.

As reported by Asa et al., an oncocytic nodule is diagnosed when more than 75% of the lesion is composed of oncocytes (59). Surgical histopathology remains the gold standard for confirming OCA, offering high diagnostic accuracy (64, 65). OCA typically presents unilaterally and is treated with hemithyroidectomy (64, 66). In contrast, OCC can be bilateral and more aggressive, necessitating total thyroidectomy (64, 66).

4.2 Low-risk/borderline tumors

4.2.1 NIFTP

This type of borderline tumor was previously referred to as noninvasive EFVPTC but was reclassified as NIFTP based on a consensus study by Thompson et al., which demonstrated its indolent biological behavior, characterized by a lack of metastasis or recurrence (15).

NIFTPs are encapsulated or well-circumscribed solid nodules, measuring up to 8 cm, with a follicular growth pattern and nuclear features typical of PTC (67–69). NIFTP accounts for approximately 9.1% of all papillary TCs and is occasionally reported in the pediatric population (70–73).

The diagnosis of NIFTP requires a surgically excised specimen, with comprehensive tumor evaluation to exclude capsular invasion (14, 15, 70). NIFTP is considered a borderline RAS-lineage tumor, situated between follicular adenoma and follicular carcinoma or invasive EFVPTC (14, 15, 70). The disease course is indolent, with excellent long-term survival following surgical excision, and lobectomy or partial thyroidectomy is usually sufficient (14, 15, 70).

According to Yang et al., the echogenicity of NIFTP can vary, ranging from markedly hypoechoic to hypoechoic, isoechoic, or mixed hypoechoic and isoechoic with cystic changes (1). Ultrasonographic characteristics of NIFTP include a wider-than-taller shape, smooth borders, occurrence in multinodular glands, and the absence of calcifications, with perinodular and intranodular vascularization. NIFTPs are ultrasonographically similar to follicular adenoma and minimally invasive follicular carcinoma (74, 75).

The inclusion criteria for NIFTP diagnosis include major features such as encapsulation or clear demarcation, a follicular growth pattern with less than 1% papillae, and a nuclear score of 2 or 3, characterized by nuclear enlargement, crowding/overlapping, elongation, irregular contours, grooves, pseudoinclusions, and chromatin clearing (14, 15, 70). Exclusion criteria include any capsular or vascular invasion, true papillary structures exceeding 1% of the tumor volume, psammoma bodies, an infiltrative border, tumor necrosis, increased mitoses, and features of other PTC variants or oncocytic lesions (14, 15, 70). Additional exclusion criteria include the presence of *BRAF V600E* and telomerase reverse transcriptase (*TERT*) promoter pathogenic variants and distant metastasis (76).

4.2.2 FT-UMP

Follicular thyroid tumor of uncertain malignant potential (FT-UMP) was first proposed by Williams et al. in 2000 and is defined as "an encapsulated or well-circumscribed tumor composed of well-

differentiated follicular cells, lacking the nuclear features of PTC, with questionable capsular or vascular invasion" (77, 78).

Ito et al. investigated the clinical characteristics of 339 patients with FT-UMP and reported that five patients (1%) experienced distant recurrence during postoperative follow-up (79). Ito et al. concluded that while FT-UMP is generally an indolent disease, some patients may experience distant recurrence, indicating the need for continued follow-up (79). However, the optimal duration of postoperative surveillance remains unclear (79).

In our study group, US imaging revealed a small, round to oval, predominantly well-defined nodule with hypo- and hyperechoic areas. In some cases, the borders of the nodules were difficult to delineate, similar to the findings reported by Ito et al. (79). Vascularization within the nodules was mixed.

Histopathological examination revealed follicular structures ranging from micro- to macrofollicles. The nodules were round to oval, with uncertain foci of capsular invasion. There were no nuclear features indicative of PTC.

4.2.3 WDT-UMP

Well-differentiated thyroid tumor of uncertain malignant potential (WDT-UMP) is a follicular neoplasm characterized by ambiguous nuclear features of PTC and questionable capsular or vascular invasion (80, 81). Most cases exhibit an indolent clinical course (77). The diagnosis is based on morphological criteria, as immunostaining is not considered reliable (77, 81). The terminology was proposed by Chernobyl pathologists to prevent unnecessary aggressive treatment (77, 81). Notably, in two children from our cohort, WDT-UMP developed following chemotherapy for ALL and after total body irradiation prior to bone marrow transplantation for chronic granulomatous disease. WDT-UMP typically presents as a well-circumscribed or encapsulated solid nodule with an excellent prognosis following lobectomy (80).

As reviewed by Wei, the differential diagnosis includes FT-UMP (a follicular neoplasm with equivocal vascular or capsular invasion but without the nuclear features of PTC) and NIFTP (a follicular neoplasm with nuclear features of PTC but without vascular or capsular invasion) (14, 80, 82).

4.3 Malignant tumors

4.3.1 Papillary thyroid carcinoma

PTC represents over 90% of all TC cases in children (9, 12, 13). Recent data from Siegel et al. indicate that TC constitutes 12% of cancers in adolescents and 2% in children under 14 years of age (83). In the United States, TC ranks as the fourth most common cancer in adolescents and the seventh most common in children (83). According to the Polish National Cancer Registry, new cases of TC in individuals under 19 years account for 2.3% of all TC diagnoses (12, 13). Among solid tumors, TC is the second most frequent in girls and the eighth in boys (12, 13).

In pediatric PTC, the most prevalent genetic alterations include RET-PTC and NTRK fusions, while pathogenic variants in BRAF V600E and RAS occur less frequently compared to adults (84, 85). Differentiated thyroid carcinomas in infancy, as reviewed by Riascos et al., are strongly associated with germline DICER1 pathogenic variants or DICER1 syndrome (47). Moreover, the presence of PDTC or thyroblastoma should prompt consideration of somatic DICER1 pathogenic variants (47).

In our cohort, US imaging revealed an irregularly contoured, hypoechogenic nodule. US characteristics indicative of thyroid malignancy include solid composition (typically hypoechoic), irregular shape and margins, a taller-than-wide configuration, microcalcifications, predominant intranodular over peripheral vascularity, rapid growth progression, and cervical lymph node enlargement (13, 20). Histopathological examination (HE) revealed a mixture of follicles of varying sizes, composed of polymorphic cells with nuclei exhibiting "glassy" clearing and grooves, penetrating the capsule.

4.3.2 Follicular thyroid carcinoma

FTC is characterized by follicular differentiation without the nuclear features of papillary carcinoma (86). FTC accounts for 6%–10% of all thyroid carcinomas (86). Clinically, FTC may develop from a preexisting adenoma. It does not typically metastasize via lymphatics but rather spreads hematogenously to the lungs, liver, bones, and brain (86). Iodine deficiency is a known risk factor. FTC is more common in female patients, comprising 75% of cases, and typically presents at an older age than papillary carcinoma, with a peak incidence between 40 and 60 years, and is rare in children (86). The etiology includes iodine deficiency, radiation exposure, and older age (86). Molecularly, FTC is associated with activation of the PI3K/AKT or RAS pathways; NRAS and HRAS mutations are present in 49% of cases, PAX8/PPARγ rearrangements in 36%, and PI3CA and PTEN mutations in 5%–10% (88–91).

As reviewed by Wei, US may reveal a solid hypoechoic nodule with a peripheral halo (indicative of a fibrous capsule); irregular or poorly defined margins may suggest malignancy (86).

According to Wei, histopathological evaluation of FTC typically reveals a trabecular or solid follicular pattern (micro-, normo-, or macrofollicular), without the nuclear features of PTC (86). Features include invasion of adjacent thyroid parenchyma, complete capsular penetration, or vascular invasion (either within or beyond the capsule) (86). The capsule is typically thickened and irregular, requiring full-thickness penetration for diagnosis (86). Vascular invasion is characterized by endothelial-covered tumor within or beyond the capsule, attached to the vessel wall or with thrombus formation. Additional findings may include nuclear atypia, focal spindle cell areas, mitotic figures, and the absence of necrosis, squamous metaplasia, psammoma bodies, or significant lymphatic invasion (86–89).

4.3.3 Poorly differentiated thyroid carcinoma

PDTC is classified under "follicular-derived carcinomas, high-grade" in the 2022 WHO classification system (2). PDTCs are malignant neoplasms of follicular cells that demonstrate limited evidence of follicular cell differentiation (90). The clinical course of PDTC lies between that of well-differentiated thyroid carcinomas (such as papillary and follicular carcinoma) and anaplastic carcinoma

(90–92). PDTC is rare in pediatric populations but more commonly affects older adults, typically between 55 and 63 years of age (93, 94). Iodine deficiency may serve as a risk factor, with some PDTCs developing *de novo* and others arising from the dedifferentiation of follicular or papillary carcinomas (95). The molecular pathogenesis of PDTC involves early events in thyroid carcinogenesis. Both *BRAF V600E*-like and *RAS*-like TCs in adults and probably also *DICER1* TCs in children can acquire additional genetic alterations—such as pathogenic variants in *TP53, TERT, CTNNB1*, and *AKT1* in adults—leading to progression toward high-grade malignancy (47, 96–99).

Clinically, PDTC often presents as a large, solitary thyroid mass, frequently associated with nodal and hematogenous metastases (96).

In our study, PDTC manifested as large, heterogeneous, hypoechoic masses on US, consistent with previous descriptions (97). As reviewed by Wei, the histologic diagnosis of PDTC, according to the Turin consensus criteria, is based on a solid/trabecular/insular growth pattern, absence of the nuclear features characteristic of papillary carcinoma, and the presence of at least one of the following: convoluted nuclei, three or more mitotic figures per 10 high-power fields, or evidence of necrosis (90).

5 Summary

This illustrative review evaluates US and histopathological features of pediatric thyroid nodules, based on cases from our tertiary thyroid center, highlighting current diagnostic challenges and approaches. Following the 2022 WHO Thyroid Tumor Classification Update, which introduced "borderline" tumor categories, treatment decisions in pediatric endocrinology have shifted toward more individualized surgical strategies, such as opting for lobectomy over total thyroidectomy in specific cases (2, 13). At our center, lobectomy patients are regularly monitored until they reach 18 years old, when they are transitioned to adult endocrine care. The optimal follow-up duration for borderline tumors, however, remains undetermined.

The 2022 European Thyroid Association guidelines underscore US as a primary tool for distinguishing benign from malignant pediatric nodules, though its sensitivity and specificity can vary significantly based on features like hypoechogenicity, calcifications, nodule shape, margin irregularity, and vascularity (13). Since most US scoring systems are derived from adult data, which does not always translate well to pediatric nodules, we apply EU-TIRADS-PL grade 5 criteria in our center, lowering the threshold diameter for FNAB from 10 to 5 mm in children (20, 21). The EU-TIRADS scales in pediatrics serve as an adjunct tool, with our clinical approach incorporating both transverse and longitudinal US views, composition analysis, and continuous monitoring of small lesions to guide FNAB decisions. Furthermore, specific patient factors—age, gender, thyroid function, and risk history, such as cancer or radiotherapy exposure—play crucial roles in management decisions.

Suspicious findings on US lead to FNAB, which remains minimally invasive, cost-effective, and highly sensitive (13, 100). Since 2015, Poland has utilized TBSRTC for categorizing cytology findings (101). A recent study by Kujdowicz et al. found that FNAB sensitivity for PTC detection was 86% in non-AIT patients but only 61.5% in AIT patients, underscoring the need for surgical intervention consideration in pediatric AIT cases with Bethesda III–VI cytology (102). This group also reported that FNAB, using TBSRTC, can identify malignancies in thyroid nodules as small as 3 mm in diameter (102). This finding underscores the system's sensitivity, particularly in pediatric cases, where smaller nodules and distinct pathological features are common.

The reclassification of NIFTP has led to updates in TBSRTC's malignancy risk estimates for indeterminate categories (15, 22). Pediatric nodules generally carry a higher malignancy risk than adult cases, highlighting the importance of adapted TBSRTC classification for children.

This review identified overlapping US features among benign, borderline, and malignant nodules, demonstrating the limitations of US as a standalone diagnostic tool. Common benign and borderline features included well-defined, oval, smooth-margined nodules, while malignant tumors more frequently exhibited marked hypoechogenicity, irregular shape and margins, and microcalcifications. Malignant nodules also grow rapidly, present extrathyroidal invasion, reinforcing histopathology's role in confirming diagnosis post-lobectomy and guiding postoperative follow-up (24).

Unique to TFND DICER1+ nodules was a cystic-solid composition, with macrocalcifications, indicating a need for genetic consultation (36).

This study's limitations include retrospective data collection from a single center and a small sample size, focusing on the most illustrative cases. However, this review adds insight by detailing pediatric low-risk tumor features and our approach to management. For borderline tumors, such as FT-UMP, comprehensive follow-up is crucial due to reports indicating a risk of distant recurrence in some cases (79). Close monitoring helps ensure early detection of any recurrence and guides timely intervention if necessary, particularly given the uncertain behavior of these lesions in pediatric populations.

Future studies may improve US-histopathological correlations and provide new insights into borderline tumor follow-up. Additionally, artificial intelligence (AI) could enhance US's role in pediatric thyroid management by simplifying risk assessments and potentially offering more personalized diagnostic outcomes, though further validation is needed to align AI models with specific patient populations (103, 104).

5.1 Conclusion

Because of the considerable overlap in sonographic features among benign, borderline, and certain malignant thyroid lesions in children, US alone is insufficient for reliable risk stratification. This overlap necessitates more frequent referrals for FNAB in pediatric patients compared to adults. Future studies incorporating advanced imaging techniques like elastography, enhanced cytopathology, and AI-driven analytics may provide new diagnostic solutions, especially given the increasing number of children presenting with solid thyroid nodules.

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 humans were approved by The Bioethics Committee of the Jagiellonian University opinion number: 118.0043.1.103.2024 issued on 19.04.2024. 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. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

DJ: Writing – original draft, Writing – review & editing. MK: Writing – original draft, Writing – review & editing. AK-W: Writing – review & editing. KK: Writing – review & editing. AT-N: Writing – review & editing. JR: Writing – review & editing. KM: Writing – review & editing. ZN: Writing – review & editing. WG: Writing – review & editing. JS: Writing – review & editing.

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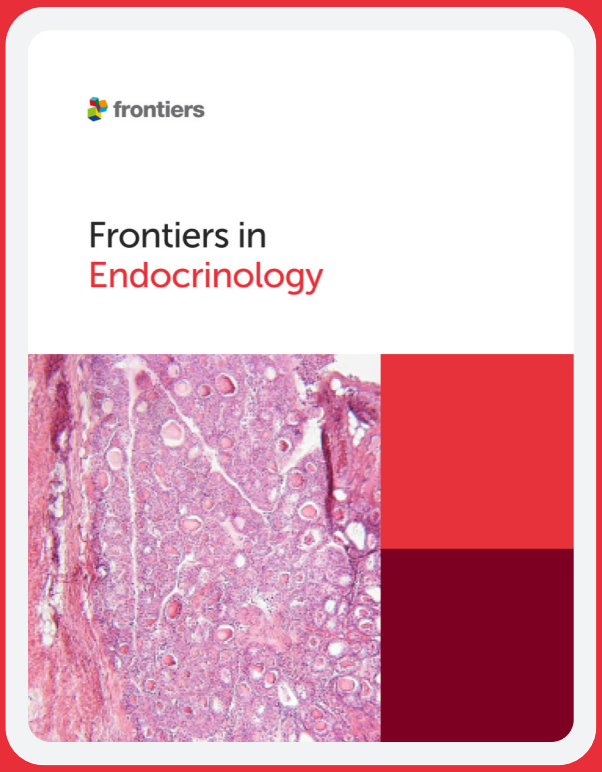

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