frontiers | Research Topics

# Insights in pediatric endocrinology 2022

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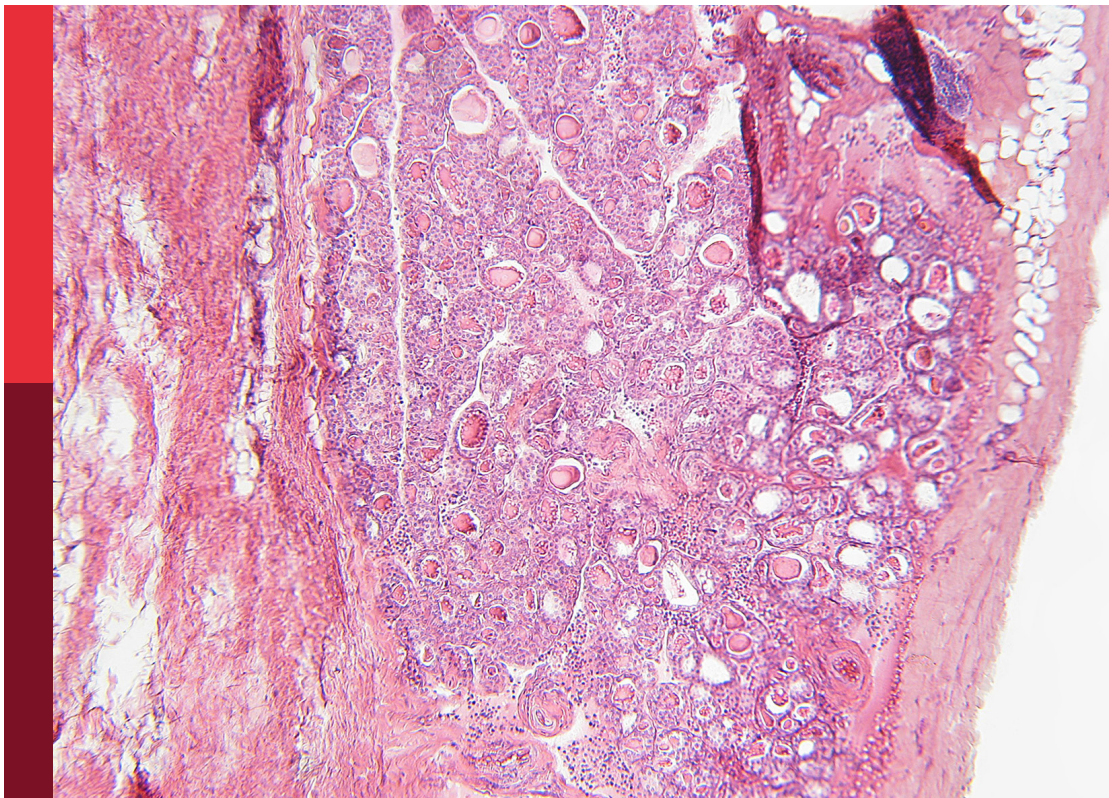

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# Insights in pediatric endocrinology: 2022

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# Editorial: Insights in pediatric endocrinology: 2022

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**pediatric endocrinology, puberty, growth, hypothalamic amenorrhea, congenital adrenal hyperplasia**

Editorial on the Research Topic
Insights in pediatric endocrinology: 2022

This special edition Research Topic was designed to highlight the progress made in Pediatric Endocrinology in the past year and to provide a thorough overview of the field. This Research Topic includes novel developments and discoveries, discusses current challenges, and provides new insights and perspectives that will guide the field into the future.

A research manuscript by Yang et al. uses chest CT to determine body composition and the relationship between the growth hormone (GH)/insulin like growth factor-1 (IGF-1) axis and muscle density in children with short stature. In a large retrospective study that included 297 children with a mean age of 10 years admitted during the COVID-19 pandemic, records were assessed for serum GH, IGF-1 levels, and two GH stimulation tests. The authors determined that peak GH is correlated with the fat mass index, and IGF-1 SDS with the skeletal muscle index. These data indicate that the GH/IGF-1 axis uses different mechanisms to regulate muscle and fat development and metabolism. The authors point to several limitations of the study. These include the cross-sectional design, inability to predict the risk of metabolic diseases, confounding variables that were not analyzed, including family income, exercise intensity, and dietary habits, and an inability to show a gender difference because the majority of children were prepubertal. Lastly, as the study was conducted in China, the findings may not be readily generalizable to other populations or ethnicities.

Although extensively used as an effective screening tool for growth hormone deficiency (GHD), bone age (BA) readings are time-consuming and can be highly variable between clinicians. Several AI systems have already been developed to assess BA yielding higher accuracy and improved time efficiency compared to manual assessment. Zhang et al. hypothesize that BA assessment gaps exist between junior and senior-level clinicians and explore the subject of using AI to assess BA in China among children with GHD. The study seeks to show that AI-assisted BA interpretation improves precision and decreases variability for junior pediatric endocrinologists during the treatment course in children with GHD. Since the classic methods used to evaluate BA were developed based on a Caucasian population (Greulich-Pyle atlas and Tanner-Whitehouse), an alternative method developed by the Chinese Bone Development Survey Group is used in the Asian population, China 05 (CH05). In the study, 290 BA radiographs from 52 children were read

by senior pediatric endocrinologists, and their consistent results were regarded as the gold standard. Two junior pediatric endocrinologists assessed the BA with and without assistance from the AI-based BA evaluation system. 20% of the images assessed by the junior pediatric endocrinologists were randomly selected and similarly re-evaluated. The performance of the junior pediatric endocrinologists improved, with the precision increasing from about 10% to over 91% using AI assistance. During GHD treatment, the longitudinal difference significantly decreased and inter-rater effect was no longer present when using AI-based BA evaluation. One of the limitations was the lack of involvement of senior pediatric endocrinologists to further validate the usage and clinical value of AI-based interpretations. Clearly, the use of AI technology will improve the precision and efficiency of BA assessments.

Mason and Rogol give a historical perspective on growth and pubertal development in children with cystic fibrosis (CF). They trace advances in pulmonary and nutritional management to improvements in the growth and development of children with CF. The authors cite multiple etiologies of impaired growth in children with CF, including malabsorption, reduced caloric intake, increased resting energy expenditure, glucocorticoid exposure, systemic inflammation, and a role for the CFTR genotype. They describe studies showing the evolution of increasing weight and height z-scores, with greater increases in weight than height. This results in some children with CF meeting criteria for overweight or obesity. The etiology is unclear and may be due to a direct effect of CFTR on the GH-IGF-1 axis. Although the literature clearly shows an association between nutritional status and pulmonary function, some recent data demonstrate an association between height in early childhood and long-term pulmonary function independent of nutritional status. They conclude with the importance of identifying factors that impact growth impairment in early life and follow growth in children receiving CFTR modulator therapy. The critical role of nutritional guidelines to optimize pulmonary function and linear growth is emphasized while also preventing obesity and its comorbidities. This is crucial at a time when early detection and modulator therapies present great promise.

Pedreira et al. focus on the role of a hypoestrogenic state of functional hypothalamic amenorrhea (FHA) on bone health. The manuscript reviews the pathogenesis of FHA induced bone changes, including the low estrogen conditions, exercise and anorexia nervosa, that result in compromised skeletal health. It also describes treatment strategies to mitigate bone loss. The sections related to the determinants of bone health in FHA are comprehensive and include contributions from the reproductive, growth, and adrenal axes and appetite-regulating hormones. The treatment strategies include a review of the controversies in the field related to whether a critical weight is necessary for the resumption of menstrual cycles. In a related section, strategies for managing bone density are discussed and include a table with a comprehensive listing of therapeutic interventions. A small part of the article is devoted to neuropsychiatric outcomes of low estrogen states, including cognitive function, emotion and mood, and eating behaviors. It finishes with a section relating to hormonal correlates.

Baskaran et al. report higher scores of anhedonic depression and anxiety in hypoestrogenic amenorrheic athletes compared with normoestrogenic eumenorrheic athletes 14-25 years old. They also demonstrate higher caudate volumes in amenorrheic vs. eumenorrheic athletes, with lower activation during reward anticipation in the right caudate in amenorrheic athletes. The latter is suggestive of a blunting of reward processing in the striatum in conditions of estrogen deficiency indicating that estrogen status may impact how we process reward.

In their review of the impact of glucose metabolism on the developing brain, Cacciatore et al. discuss studies that demonstrate deleterious effects of both hypoglycemia and hyperglycemia on brain development, cognitive function (including an impact on intelligent quotient, learning, memory and executive function). This is because glucose is an essential substrate for brain development and functioning. They also review functional MRI data, including findings of alterations in brain structure and function even after a single episode of diabetic ketoacidosis.

There is a dearth of information on biomarkers for complications of type 1 diabetes mellitus in children. Gong et al. describe a novel biomarker, alpha-klotho, for diabetic nephropathy, a major cause of end-stage renal disease, in children with type 1 diabetes mellitus. Although alpha-klotho (KL), a co-receptor for fibroblast growth factor (FGF) 23, which is regulated by the miRNA miR-192, is lower in mouse models and adults with chronic kidney disease, data are limited in children. The investigators studied 79 pediatric patients with type 1 diabetes for 7.2 ± 3.9 years with a 2-year average HbA1c of 8.0 ± 1.3. They found that KL was inversely correlated with diabetes duration and HbA1c, indicating its potential role in glycemic control. Serum miR-192 was negatively associated with KL among children with a prolonged duration of diabetes (≥12 years). A mechanistic approach to understanding the role of miR-192 and KL expression in a cell culture model demonstrated overexpression of miR-192, downregulated cellular KL mRNA and protein levels as well as decreased KL levels in the media. Using a reporter assay, a miR-192 mimic reduced the activity of a reporter. Additional studies showed an increase in oxidative stress and expression of inflammatory and senescence markers. These data suggest that KL is a direct target of miR-192 and implicate oxidative stress as the mechanism of renal disease. They conclude that miR-192 and/or KL levels could serve as early biomarkers for diabetic nephropathy in children with type 1 diabetes.

This Research Topic includes three papers on precocious puberty and one on congenital adrenal hyperplasia. Prosperi and Chiarelli review data from multiple studies from Italy, China, Turkey and India that report an increase in cases of central precocious puberty and rapidly progressive puberty during the COVID-19 pandemic, sometimes, but not always related to an increase in BMI from the more sedentary lifestyle during the lockdown. Most papers do not provide documentation of SARS-COV-2 infection in children presenting with early or rapidly progressive puberty, and the authors speculate whether there may be a direct effect of the virus, or an indirect effect (through psychological factors) on pathways regulating GnRH secretion.

The review by Calcaterra et al. addresses the 'hot topic' of the gut microbiome and sex steroids, and they explore interactions, potentially bidirectional, between the gut microbiome and sex hormones, differences in boys vs. girls during pubertal development and during female precocious puberty. Presently, the evidence on the interaction between gut microbiota and sex hormones remains limited in pediatric patients. The authors begin by reviewing the basic principles of puberty and precocious puberty, followed by a summary of the role of the microbiome in known health and disease states. They focus on known associations between the gut microbiome and obesity and relate this to the earlier puberty seen in obese children. They then explore known interactions between the gut microbiome and sex steroids, referred to as the sex-hormone-gut microbiome. Several manuscripts are referenced, which provide evidence that the diversity of the gut microbiome changes through pubertal development and alterations may occur in girls with central precocious puberty (CPP). The authors call for additional research to increase our understanding of the relationship between sex hormones and the gut microbiome. They summarize by stating that further clarification of the interactions between sex hormones and the gut microbiome may lead to microbiota-targeted therapies in pubertal disorders.

Yoo et al. report on the efficacy and safety of the 22.5 mg 6-monthly triptorelin pamoate formulation in suppressing puberty and improving adult height prediction in 33 girls and 9 boys with central precocious puberty. Six-monthly depot gonadotropin releasing hormone analogs allow clinic visits to be spaced out for patients with central precocious puberty and reduce patient burden. Itonaga and Hasegawa review current knowledge regarding monitoring of treatment of pediatric patients with 21-hydroxylase deficiency, and discuss the pros and cons of biochemical serum (17-hydroxyprogesterone, androstenedione and ACTH) and urine (pregnanetriol and other steroid metabolites) testing. At this time serum testing is preferred because it is less expensive, more standardized, and well-established in clinical care.

Finally, Dacal et al. present interesting data from prepubertal and pubertal boys with hematological malignancies before initiation of chemotherapy and show lower levels of inhibin B and anti-Mullerian hormone with low or inappropriately normal FSH levels compared to a reference population, with lesser involvement of the LH-Leydig cell compartment (12). Following three months of chemotherapy, the authors report high levels of FSH and LH levels with persistently low levels of inhibin B and anti-Mullerian hormone, but normalization of testosterone. The authors posit occurrence of a primary testicular dysfunction with an associated functional central hypogonadism before treatment initiation (when general markers of health status are also low), with improvement in hypothalamic-pituitary function (but persistence of testicular dysfunction) following three months of chemotherapy, when markers of general health are improved.

The Research Topic thus covers a range of novel topics and comprehensive reviews addressing important issues in the field of pediatric endocrinology today. As always, more research is necessary to answer hitherto unanswered questions, and we hope that perusal of this Research Topic stimulates ideas for future research, including collaborative research.

## Author contributions

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

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# Association Between the Growth Hormone/Insulin-Like Growth Factor-1 Axis and Muscle Density in Children and Adolescents of Short Stature

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**Objective:** To evaluate the association between the growth hormone (GH)/insulin-like growth factor-1 (IGF-1) axis and muscle density in children and adolescents of short stature.

**Methods:** Participants were children and adolescents of short stature hospitalized in the Affiliated Hospital of Jining Medical University between January 2020 and June 2021. All participants had CT scan images available. We performed an analysis of the images to calculate the muscle density or skeletal muscle attenuation (SMA), skeletal muscle index (SMI), and fat mass index (FMI). Bioelectrical impedance analysis (BIA) was used to ensure that chest CT is a credible way of evaluating body composition.

**Results:** A total of 297 subjects were included with the mean age of 10.00 ± 3.42 years, mean height standard deviation score (SDS) of -2.51 ± 0.53, and mean IGF-1 SDS of -0.60 ± 1.07. The areas of muscle and fat tissues at the fourth thoracic vertebra level in the CT images showed strong correlation with the total weights of the participants ($R^2$ = 0.884 and 0.897, respectively). The peak of GH was negatively associated with FMI (r = - 0.323, P <.01) and IGF-1 SDS was positively associated with SMI (r = 0.303, P <.01). Both the peak GH and IGF-1 SDS were positively associated with SMA (r = 0.244, P <.01 and r = 0.165, P <.05, respectively). Multiple stepwise linear regression analysis demonstrated that the GH peak was the predictor of FMI (β = - 0.210, P < .01), the IGF-1 SDS was the predictor of SMI (β = 0.224, P < .01), and both the peak GH and IGF-1 SDS were predictors of SMA (β = 0.180, P < .01 and β = 0.222, P < .01).

**Conclusions:** A chest CT scan is a credible method of evaluating body composition in children and adolescents of short stature. In these patients, peak GH and IGF-1 SDS are independent predictors of muscle density and the GF/IGF-1 axis may regulate body composition through complex mechanisms.

**Keywords: short stature, muscle density, GH/IGF-1 axis, body composition, GDDSD study**

# INTRODUCTION

Growth hormone (GH) promotes linear growth and plays key role in regulating muscle development and metabolism. Insulin-like growth factor-1 (IGF-1) is the major mediator by which GH elicits skeletal muscle cell proliferation and myocyte differentiation (1, 2). Children and adolescents of short stature often have increased fat mass and reduced lean mass and muscle strength. This phenomenon is more distinct in those with severe growth hormone deficiency (GHD) (3–5). Treatment with GH can increase muscle mass and strength and decrease fat tissue percentage. Discontinuing it leads to a reversal of these effects (4, 6–10).

Muscle density is an important parameter of muscle health and is emerging as a predictive factor for various metabolic diseases. In adults, low muscle density is associated with a high risk of diabetes, cardiovascular diseases, bone fractures, and worse outcomes in patients with cancer and other critical illnesses (11–18). Several studies have demonstrated that muscle density is a predictor of bone density, bone strength, and cardio-metabolic risk in children and adolescents (19–21). Currently, computed tomography (CT) is the gold standard for investigating qualitative changes in muscles. Low muscle attenuation indicates a high proportion of myosteatosis (intermuscular and intramuscular fat infiltration); whereas high muscle attenuation indicates low muscle fat infiltration (high muscle density) (16, 22, 23).

Although previous studies have reported that GH plays an important role in maintaining muscle mass, none have investigated the role of GH on muscle density. In the past two years, a chest CT scan was performed on some children and adolescents of short stature admitted to our hospital during the COVID-19 pandemic. We performed this retrospective study to evaluate the relationship between the GH/IGF-1 axis and muscle density in children and adolescents of short stature. The areas of skeletal muscle and fat at the fourth thoracic vertebra (T4), assessed by CT, and the total weight assessed by bioelectrical impedance analysis (BIA) were correlated to ensure chest CT was a credible method of evaluating body composition.

# METHODS

## Study Patients

All the subjects enrolled were in the GDDSD study (http://www.chictr.org.cn, ChiCTR1900026510), an ongoing prospective, observational, open cohort study that is evaluating the etiology of growth and development diseases and the long-term safety and effectiveness of growth hormone therapy in a real-life clinical setting (24). Children and adolescents of short stature in the study were those hospitalized between January 2020 and June 2021 in the Department of Endocrinology of the Affiliated Hospital of Jining Medical University and had a chest CT scan done. Short stature is defined as a condition in which the individual's height is two standard deviations (SD) or more below the population mean for the relevant age and gender (25). The exclusion criteria were as follows: (1) patients missing the values of IGF-1 and GH stimulation test; (2) patients with chronic disease, malignant tumors, and abnormal thyroid function; and (3) patients with conditions such as skeletal dysplasia, achondroplasia, and disorders of sex development. Approval was obtained from the Ethics Committee of the Affiliated Hospital of Jining Medical University and informed consent forms were signed by all the participants' parents.

## Body Composition Measurements

Two authors of this study identified axial CT images at the T4 level and used them to calculate the skeletal muscle area, subcutaneous fat area, and mean skeletal muscle attenuation (SMA). The Slice-O-Matic software (V.5.0, TomoVision, Montreal, Quebec, Canada) was used in this analysis and the attenuation threshold was set to −29 to 150 Hounsfield units (HU) for skeletal muscle, and −190 to −30 HU for subcutaneous adipose tissue. Each type of tissue found in the T4 CT images was shaded with a different color that corresponded to these thresholds. The T4 cross-sectional skeletal muscle area (T4MA) and subcutaneous fat area (T4FA) were recorded in $cm^2$ and the SMA in mean HU. The skeletal muscle index (SMI) and fat mass index (FMI) were calculated by dividing the skeletal muscle and subcutaneous fat area in $cm^2$ by height in $m^2$. The total muscle mass (TMW) and total fat tissue mass (TFW) were measured using BIA with patients in a fasting state.

## Laboratory Measurements

Overnight fasting blood samples were collected from all participants and laboratory parameters were measured using methods described in a study we did previously (24). The biochemical and immune indices used in this study include GH, IGF-1, IGF-binding protein-3 (IGFBP-3), hemoglobin (Hb), alanine aminotransferase (ALT), albumin (ALB), creatinine (Cr), triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL-C), low-density lipoprotein (LDL-C), blood calcium (Ca), blood phosphate (P), free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (TSH), luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The IGF-1 SD score (SDS) was calculated using the reference values in healthy children of the same age and sex (26). Two of three GH stimulating tests were performed to evaluate the peak level of GH (levodopa, 500 mg for those ≥ 30 kg, 250 mg for those < 30 kg and ≥ 15 kg, and 125 mg for those < 15 kg; insulin, 0.1-0.15 U/kg; and arginine, 0.5 mg/kg). Blood samples were collected at 0, 30, 60, 90, and 120 minutes to obtain serum GH concentrations at each of these points.

## Statistical Analysis

Continuous variables were summarized using the median and IQR for non-normally distributed data and the mean ± SD for normally distributed data. Categorical variables were summarized as the frequency count in percentage. Correlations between variables were assessed by Pearson's correlation coefficient. Multiple stepwise linear regression analysis was

used to identify independent factors associated with muscle density. Statistical analysis was performed using SPSS software (26.0; IBM, Armonk, NY). A $P$ value less than 0.05 was considered statistically significant.

## RESULTS

A total of 297 eligible participants (189 males and 108 females) were included. The mean age was 10.00 ± 3.42 years (ranging from 4 to 16 years). Their mean height SDS was −2.51 ± 0.53 and the mean IGF-1 SDS was -0.60 ± 1.07. The body compositions measured by BIA and chest CT and other parameters are summarized in **Table 1**.

### The Relationship Between CT Scan and BIA in Measuring Body Composition

Scatter plots show the T4MA and T4FA assessed by chest CT and TMW and TFW assessed by BIA (**Figure 1**). The T4MA showed a strong correlation with TMW (TMW = 0.14 T4MA − 2.56; $R^2$ = 0.884; $P$ <.001) and T4FA showed a strong correlation with TFW (TFW = 0.11 T4FA + 0.72; $R^2$ = 0.897; $P$ <.001).

### The Correlation Between the GH/IGF-1 Axis and CT Scan Body Composition

Male patients had a higher level of SMI (60.48 ± 8.93 and 56.79 ± 8.10, $P$ <.01) compared with female patients, but there were no differences in FMI and SMA. The peak GH was negatively associated with FMI (r = - 0.323, $P$ <.01) and positively associated with SMA (r = 0.244, $P$ <.01). The level of IGF-1 SDS was positively associated with SMI (r = 0.303, $P$ <.01) and SMA (r = 0.165, $P$ <.05). The correlations between body composition and other clinical factors are shown in **Table 2**.

Multiple stepwise linear regression analyses of variables related to the SMI, FMI and SMA are listed in **Table 3**. After adjusting for confounding factors, the peak GH was the predictor of FMI (β = - 0.210, $P$ < .01) and SMA (β = 0.180, $P$ < .01); IGF-1 SDS was the predictor of SMI (β = 0.224, $P$ < 0.01) and SMA (β = 0.222, $P$ < .01).

## DISCUSSION

In this study, the peak GH and IGF-1 SDS are positively correlated with muscle density in children and adolescents of short stature. After adjusting for confounding factors, both the peak GH and IGF-1 SDS are independent predictors of muscle density. To the best of our knowledge, this is the first study that demonstrates the relationship between the GH/IGF-1 axis and muscle density.

Several technologies including ultrasonography, dual x-ray absorptiometry, BIA, CT, and magnetic resonance imaging could be used to assess body composition (27). Among these, CT scans quantify bone mineral density, visceral and subcutaneous fat, skeletal muscle, liver fat, and arterial vascular calcification. Thus, they are the most comprehensive modality (28). The predictive value of a chest CT in whole-body composition has been evaluated in healthy adults or patients with cancer. The cross-sectional areas of muscle and fat tissue have shown a moderate correlation with total body weight (29–31). In this study, most of the participants are prepubertal or adolescent, and there is little interference from the abdomen, hip, and limbs. Our research demonstrated that the areas of muscle and fat tissues at the T4 level assessed by chest CT highly correlated with the total weights assessed by BIA ($R^2$ = 0.884 and 0.897, respectively). This provides a possibility of assessing body composition incidentally in some children if chest CT is required for their diagnosis and treatment.

Children and adolescents of short stature, especially those with GHD, often have increased fat mass and reduced lean mass and muscle strength. In 2016, Improda et al. summarized the role of the GH/IGF-1 axis in the muscle and skeletal health of children and adolescents (32). In general, childhood-onset GHD can affect bone and muscle mass and strength, and GH replacement therapy has beneficial effects. Moreover, GH withdrawal at final height can result in reduced bone and muscle mass, potentially leading to increased fracture risk in adulthood (32). Our study also confirmed the association between the GH/IGF-1 axis and body composition. The peak GH is correlated with FMI and IGF-1 SDS is correlated with SMI.

**TABLE 1** | Characteristics of patients included in this study.

| Characteristic | Patients | Value | Characteristic | Patients | Value |
|---|---|---|---|---|---|
| Age (years) | 297 | 10.00 ± 3.42 | Hb (g/L) | 296 | 131.30 ± 11.04 |
| Bone age (years) | 289 | 8.43 ± 3.83 | ALT (U/L) | 296 | 14.70 ± 8.07 |
| Peak of GH (ng/mL) | 297 | 7.29 ± 5.00 | ALB (g/L) | 296 | 46.88 ± 2.86 |
| IGF-1 SDS | 297 | -0.60 ± 1.07 | Cr (umol/L) | 295 | 43.82 ± 11.66 |
| IGFBP-3 (μg/mL) | 293 | 4.55 ± 1.31 | TG (mmol/L) | 282 | 0.68 (0.53,0.91) |
| High SDS | 297 | -2.51 ± 0.53 | TC (mmol/L) | 282 | 3.91 ± 0.75 |
| Weight (kg) | 297 | 27.94 ± 11.91 | HDL (mmol/L) | 282 | 1.52 ± 0.41 |
| BMI SDS | 297 | -0.22 ± 1.53 | LDL (mmol/L) | 282 | 2.21 ± 0.52 |
| SMA (HU) | 297 | 47.54 ± 4.05 | Ca (mmol/L) | 296 | 2.45 ± 0.10 |
| T4MA ($cm^2$) | 297 | 94.85 ± 31.63 | P (mmol/L) | 296 | 1.60 ± 0.16 |
| T4FA ($cm^2$) | 297 | 33.83 (22.57,58.23) | FT3 (pmol/L) | 294 | 6.88 ± 2.26 |
| SMI ($cm^2/m^2$) | 297 | 59.03 ± 8.83 | FT4 (pmol/L) | 294 | 17.90 ± 3.01 |
| FMI ($cm^2/m^2$) | 297 | 23.99 (15.59,37.22) | TSH (mIU/L) | 294 | 2.66 ± 1.36 |
| TMW (kg) | 205 | 10.88 ± 4.64 | LH (mIU/ml) | 281 | 0.23 (0.01,1.57) |
| TFW (kg) | 205 | 4.90 (2.90,7.95) | FSH (mIU/ml) | 281 | 2.45 (1.05,4.36) |

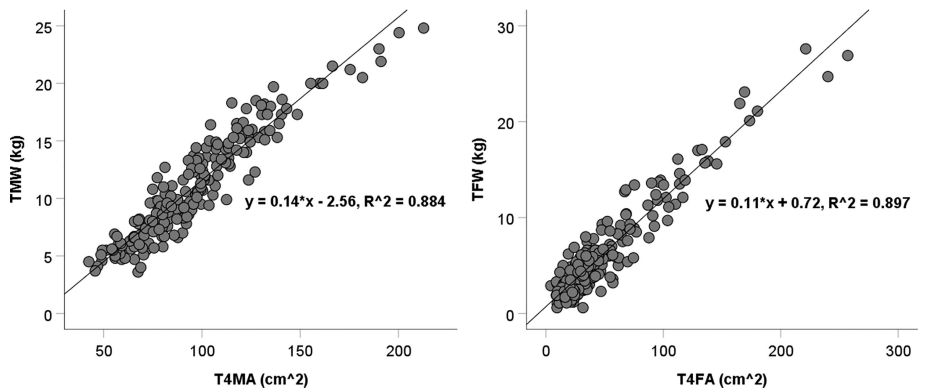


**FIGURE 1** | Scatter diagram of T4MA and T4FA with TMW and TFW. TMW, total muscle weight; TFW, total fat weight; T4MA, T4 muscle area; T4FA, T4 fat area.

**TABLE 2** | Correlations between body composition evaluated by T4 CT scan and clinical factors.

| Variables | SMI ($cm^2/m^2$) | FMI ($cm^2/m^2$) | SMA (HU) |
|---|---|---|---|
| Age (years) | - 0.027 | 0.195* | - 0.229** |
| Bone age (years) | 0.036 | 0.234** | - 0.217** |
| High SDS | 0.028 | - 0.091 | 0.108 |
| Weight (kg) | 0.198* | 0.535** | - 0.338** |
| BMI SDS | 0.293** | 0.563** | - 0.222** |
| Peak of GH (ng/mL) | 0.071 | - 0.323** | 0.244** |
| IGF-1 SDS | 0.303** | 0.069 | 0.165* |
| IGFBP-3 (μg/mL) | 0.054 | 0.132* | - 0.085 |
| Hb (g/L) | 0.216** | 0.098 | 0.075 |
| ALT (U/L) | 0.101 | 0.404** | - 0.222** |
| ALB (g/L) | 0.067 | 0.137* | - 0.060 |
| Cr (μmol/L) | 0.177* | 0.053 | 0.073 |
| TG (mmol/L) | 0.135* | 0.210** | 0.005 |
| TC (mmol/L) | - 0.144* | 0.080 | - 0.129* |
| HDL (mmol/L) | - 0.005 | - 0.076 | 0.002 |
| LDL (mmol/L) | - 0.026 | 0.289** | - 0.114 |
| Ca (mmol/L) | 0.049 | 0.112 | - 0.042 |
| P (mmol/L) | - 0.057 | - 0.043 | 0.078 |
| FT3 (pmol/L) | 0.127* | 0.136* | - 0.041 |
| FT4 (pmol/L) | - 0.013 | - 0.105 | 0.039 |
| TSH (mIU/L) | 0.029 | 0.058 | 0.004 |
| LH (mIU/mL) | 0.055 | 0.124* | - 0.103 |
| FSH (mIU/mL) | 0.008 | 0.214** | - 0.175* |

*Correlations are shown with the coefficient r value. * P < 0.05; ** P < 0.01.*

This indicates that the GH/IGF-1 axis uses different mechanisms in the regulation of muscle and fat development and metabolism. Unlike skeletal muscle cell proliferation and myocyte differentiation by GH, which are almost entirely mediated by IGF-1, adipose tissue lipolysis appears to be directly mediated *via* the GH receptor (33–35). The body composition, in turn, also affects the levels of the peak GH and IGF-1. For example, obesity reversibly suppresses GH secretion driven by elevated free fatty acids, whereas IGF-I levels remain normal or elevated due to elevated portal insulin levels (36). Therefore, there are bidirectional associations exist between the GH/IGF-1 axis and body composition.

It is well known that adults of short stature or GHD are at a higher risk of hypertension, dyslipidemia, cardiovascular disease, type 2 diabetes, and fracture (37–42). Coincidentally, individuals with lower muscle attenuation on CT also have a higher risk of these metabolic diseases (15, 16, 43–47). It is possible that patients of short stature already have impaired muscle density in their childhood and the GH/IGF-1 axis plays a critical role in muscle density regulation. Our study demonstrated that both the peak GH and IGF-1 SDS are independent predictors of SMA in children and adolescents of short stature. SMA is a comprehensive marker that determines both SMI and FMI, and the GH/IGF-1 axis may regulate muscle density through complex mechanisms (48, 49).

**TABLE 3** | Multiple stepwise linear regression analysis of factors associated with SMI, FMI and SMA.

| SMI ($cm^2/m^2$) | | FMI ($cm^2/m^2$) | | SMA (HU) | |
|---|---|---|---|---|---|
| **Variables** | **β value** | **Variables** | **β value** | **Variables** | **β value** |
| IGF-1SDS | 0.224** | BMI SDS | 0.403** | Peak of GH (ng/ml) | 0.180** |
| BMI SDS | 0.278** | ALT (U/L) | 0.234** | Age (years) | - 0.264** |
| Gender (male) | 0.210** | Peak of GH (ng/mL) | - 0.210** | IGF-1SDS | 0.222** |
| TC (mmol/L) | - 0.110* | LDL (mmol/L) | 0.203** | BMI SDS | - 0.186** |
| – | – | Bone age (years) | 0.186** | TC (mmol/L) | - 0.132* |
| – | – | FSH (mIU/mL) | 0.109* | ALT (U/L) | - 0.134* |
| - | - | - | - | - | - |

*Adopted factors: gender, BMI SDS, IGF-1SDS, Hb, Cr, TG, TC and FT3 for SMI; age, bone age, BMI SDS, the peak of GH, IGFBP-3, ALT, ALB, TG, LDL, FT3, LH and FSH for FMI; age, bone age, BMI SDS, the peak of GH, IGF-1SDS, ALT, TC and FSH for SMA. * P < 0.05; ** P < 0.01.*

Our study has several limitations. First, the cross-sectional design of the study does not allow for causal inference and is limited in clarifying the underlying pathophysiological mechanisms involved. Second, to determine whether lower muscle density in childhood can predict higher risks of metabolic diseases, a long-term follow-up is required. Third, some confounding factors that might influence muscle health such as family income, exercise intensity, and dietary habits were not included in the analysis. Fourth, most of the participants enrolled in our study were prepubertal and only SMI showed a gender difference in them. We did not perform subgroup analyses like those done in adult studies. Lastly, our study was conducted in China and the findings may not be readily generalizable in other populations or ethnicities.

In conclusion, this study confirmed the credibility of chest CT in evaluating body composition in children and adolescents of short stature. In these patients, both the peak GH and IGF-1 SDS are independent predictors of muscle density; and the GH/IGF-1 axis may regulate body composition through complex mechanisms.

## DATA AVAILABILITY STATEMENT

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

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee of the Affiliated Hospital of Jining Medical University (2019C003, Jining, China). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

The manuscript was conceived by QY and FL, with manuscript questions and analytic plan designed by GY, QY, BB, and FL. FL wrote the manuscript, interpreted the data, critically reviewed and revised the manuscript. GY, QY, MZ, and BB contributed to writing, data analysis, data interpretation, critical review and revision. YL, YZ, SC, and DH contributed to data interpretation, critical review and revision. All authors had access to the data and all authors agreed to submit the final manuscript. FL was supported by the Jining Key Research and Development Projects. GY, QY, and FL are the guarantors of this work and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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# Trends in Growth and Maturation in Children with Cystic Fibrosis Throughout Nine Decades

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Since cystic fibrosis (CF) was first described in 1938, there have been many discoveries and innovations in the field, each having a profound impact on survival, growth and quality of life. For example, the introduction of enteric-coated pancreatic enzyme microspheres increased fat absorption and improved nutritional status. Early detection of CF through newborn screening facilitated prompt nutritional intervention for infants at high risk of malnutrition. Use of anti-pseudomonal therapy, such as inhaled tobramycin, increased weight gain and pulmonary function in addition to reducing pulmonary exacerbations. Similarly, DNAse and hypertonic saline improved pulmonary function and reduced exacerbations. The identification of the *CFTR* gene and its protein product were fundamental in understanding the pathophysiology of CF and paved the way for advances in both diagnosis and management. In fact, CFTR modulator therapies have revolutionized the care for individuals with CF. Here, we examine the impact of these interventions on the nutritional status, growth and pubertal maturation of children and adolescents with CF.

**Keywords: cystic fibrosis, growth, height, weight, puberty, body composition**

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## INTRODUCTION

Cystic Fibrosis (CF) affects nearly 70,000 individuals worldwide. It is caused by an autosomal recessive mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which results in a dysfunctional CFTR protein. This, in turn, impairs chloride ion transport to the cell surface, resulting in viscous secretions in the lungs, pancreas, intestine and hepatobiliary ducts, leading to obstruction and fibrosis (1). Since its initial description by Dorothy Andersen in 1938 (2), there have been many landmark discoveries that have led to remarkable improvements in life expectancy and quality of life.

For decades, chronic infection, malabsorption of essential nutrients, inflammation and the frequent use of corticosteroids set the child with CF on a course of diminished weight gain and linear growth restriction. It should be noted that increased energy requirements (expenditure) due to inflammation and chronic pulmonary disease also contribute to the energy deficit. Growth restriction may even begin *in utero* since the birthweight of children with CF is approximately 250 g less than that of healthy newborns (3). It may be that the absent CFTR affects placental function.

Over the decades the treatment goals have been to optimize pulmonary function and growth through proper nutrition and reduced inflammation. We aim to characterize the trends in growth

and maturation over time. Notably, many studies report anthropometric data differently, using weight or height-for-age or weight-for-height as a % or z-score or % of reference median. In their study of 13,116 children with CF, Lai et al. discovered inconsistencies in classifications when using various criteria, underscoring the importance of standardized definitions (4).

Nevertheless, it is evident that interventions such as multi-disciplinary care and the introduction of newborn screening have contributed to the increased growth of children with CF, demonstrated by an increase in the average height z-score at age 6 years from -0.69 to 0.39 SD (5).

## THE 1930'S

Cystic Fibrosis was first described in 1938 by Dorothy Andersen who carefully reviewed the clinical histories and post-mortem examinations from 49 individuals with pancreatic fibrosis, many of whom died during infancy or early childhood (2). Infants who died within the first week of life were felt to have died from intestinal obstruction. The remaining children were characterized by failure to gain weight beginning in the neonatal period, hunger, distended abdomens, intolerance of dietary fat with large fatty stools in the absence of vomiting and diarrhea, and chronic respiratory tract infections. Andersen termed the condition cystic fibrosis of the pancreas based on the observation that the pancreatic acinar tissue "was replaced by epithelium-lined cysts containing concretions and surrounded by fibrous tissue"; there was also evidence of vitamin A deficiency. The lungs demonstrated "bronchitis, bronchiectasis, pulmonary abscesses arising in the bronchi", and/or lobular pneumonia with S. aureus as a common "bacteriologic agent". The oldest child in the cohort was 14.5 years (2). In 1935 Parmelee described this girl's stature as closely approximating normal until age 11, after which time her growth was described as "retarded", but the "development of secondary sex characteristics was not." Her weight was reported to be considerably below average throughout much of her childhood and adolescence (6). Based on the findings in this report, this child likely had more mild disease.

## THE 1940'S

Anthropometric data in children with CF throughout the 1940's are largely unavailable.

## 1950'S

Given the short life span in those diagnosed with CF in the 1930's and 1940's, there are few data on growth and puberty for these children. However, the life expectancy in the 1950's (1951-1956), increased to 59 months (7) allowing for more detailed descriptions of growth and nutritional status.

Rustin McIntosh, for example, recorded observations on a cohort of 23 patients with CF who survived to at least 10 years of age. In this group of children, malnutrition occurred more commonly during infancy compared to childhood, but height and weight were "retarded" (average height z-score 2 SD below mean and average weight 1.8 SD below mean). Height and weight were noted to increase in response to appropriate therapy for staphylococcal infections, suggesting that chronic infection and inflammation play a role in the poor growth observed in children with CF (8).

Several years later, Shwachman and Kulczycki, described a larger cohort of 105 children who were followed longitudinally for at least 5 years after diagnosis. The majority (87/105) received antibiotic therapy. Their patient population consumed a liberal diet high in protein with limited fat intake. Those who had pancreatic insufficiency took pancreatic enzyme replacement with each meal and double the typical daily dose of multivitamins. The patients were divided into groups based on the age of diagnosis and each was assigned a score based on their level of tolerated activity, physical and radiographic findings and nutritional status (Shwachman-Kulczycki or the SK score). The nutritional score was based on height and weight percentiles for age, stool characteristics, muscle mass and tone, and degree of abdominal distension. The nutritional score was not reported for all subgroups but was documented for the group diagnosed between the age of 7 and 16 years both at baseline and at follow-up. At baseline, none of the 9 children with CF were categorized as marked malnutrition, 4/9 (45%) had a weight and height for age under the 3$^{rd}$ centile and 2 (22%) had height and weight for age over the 25$^{th}$ centile. At follow-up 5 to 10 years later, 2 (22%) were categorized as having marked malnutrition, 4/9 (44%) had a weight and height for age less than the 3$^{rd}$ centile and none had a height and weight for age above the 25$^{th}$ centile, suggesting a decline in nutritional status and linear growth over time (7) (**Table 1**).

The birthweights of infants with CF were significantly below those of both the general population (30) and unaffected siblings (31), suggesting that the slow growth was not solely due to infections, pancreatic insufficiency or nutritional status, but began *in utero*.

## THE 1960'S

The 1960's marked the beginning of a specific focus on growth in children with CF. Sproul and Huang studied the growth patterns in 50 children and noted a period of accelerated weight gain after initiation of therapy that included antibiotics and pancreatic enzyme replacement therapy (PERT), with the longest duration of effect occurring when therapy was initiated during infancy. They did not find an effect on linear growth despite a minimum observation period of 2 years. The authors observed that the median height and weight for all age groups were under the 10$^{th}$ centile with weight more affected than height in the pre-school and school-age children and growth "retardation" more prominent in the preadolescent and adolescent age groups, a

**TABLE 1** | Growth Data over Time.

| Authors | Year | Location | Study Details | Findings |
|---|---|---|---|---|
| Shwachman (7) | 1958 | US | Longitudinal study over 5-14 yrs<br>n=105 (Total) age 5 to >15 yrs<br>n=9 (Number with nutritional score at baseline & 5-10 years later) | • Malnourished: 0% to 22%<br>• Wt & ht <3%: 45% to 44%<br>• Wt & ht >25% 22% to 0% |
| Sproul (9) | 1964 | US | Longitudinal study over ≥ 2 yrs<br>n=50 infancy through adolescence | • Ht and wt <10%<br>• ■ Wt affected > ht in young; stunting of ht & wt with age<br>• ↑ Wt after Abx and PERT (no impact on linear growth) |
| Kreiβl (10) | 1972 | Germany | Longitudinal study over 1-12 yrs<br>n=53 aged 2-13.8 yrs | • Ht 25-50%<br>• Wt affected > ht (M 25%; F 3-10%) |
| Berry (11) | 1975 | US | Case-control study of 'nutritional supplement'<br>n=63 | • ↑ Wt to -1SD w/early dx<br>• ↓ WV > HV after 8 yrs |
| Mitchell-Heggs (12) | 1976 | UK | Longitudinal study over 1.1-14.25 yrs<br>n=45 aged 12-27 yrs | • Ht M 25%; F 25-50%<br>• Wt affected > ht<br>• ■ 47% wt <3% |
| Corey (13) | 1988 | US & Canada | Cross-sectional study<br>n=1,033 aged 0-45 yrs | • Pts Toronto taller than Boston (M 42% vs. 33% p<0.001; F 44% vs 33%)<br>• M Toronto heavier than Boston<br>• Attributed to diff in dietary fat & PERT |
| Keller (14) | 2003 | Switzerland | Longitudinal study for at least 11 yrs<br>n=75<br>(40 born 1968-80 & 35 born 1982-96) | • BMI sig ↓in young kids born earlier (p<0.047 for 2 yo; p=0.0045 for 5 yo) |
| Nir (15) | 1996 | Denmark | Cross-sectional study<br>n=223 aged 0.75-41 yrs | • Ht -0.46 SD<br>• Wt M -0.6 SD; F -0.7 SD<br>• BMI ↓ over time (98% in younger pts 83-90% in adult) |
| Farrell (16) | 1997 | US | Randomized prospective longitudinal study (Wisconsin CF Neonatal Screening Project)<br>n=96 from age at diagnosis through 10 yrs | • Length/ht & wt ↑ in those dx by NBS than non-screened<br>• ■ Ht -0.2 vs. -1.2; p<0.001<br>• ■ Wt -0.5 vs -1.2; p=0.008<br>• ■ Persists over 10 yrs (wt p=0.04; ht p=0.02) |
| Lai (4) | 1998 | US | Cross-sectional study<br>n=13,116 | • Ht 30%<br>• Wt 20% |
| Lai (17) | 2000 | US | Randomized prospective longitudinal study (Wisconsin CF Neonatal Screening Project)<br>n=82 from age at diagnosis through 10 yrs | • Ht & wt near nml in those dx by NBS (without MI) up to age 13<br>• ■ Ht -0.06 SD<br>• ■ Wt -0.02 SD |
| Farrell (18) | 2001 | US | Randomized prospective longitudinal study (Wisconsin CF Neonatal Screening Project)<br>n=104 from age at diagnosis through 13 yrs | • Risk for wt or ht <10% ↓ those dx by NBS than non-screened (OR 4.12 wt & 4.62 ht) |
| Kastner-Cole (19) | 2005 | UK | Cross-sectional study<br>n=2,987 aged 0.8 to 55.7 yrs | • BMI M -0.28 to 0.8 SD; F -0.28 to 0 SD<br>• ■ 10.2% OW or OB<br>• ■ BMI ↓ late childhood/adolescence vs. infancy/early childhood |
| Hanna (20) | 2015 | US | Cross-sectional study<br>n=226 aged 2-18 yrs | • Malnourished (BMI <10%): 7%<br>• At risk (BMI 10-25%): 12%<br>• Healthy (BMI 25-85%): 57%<br>• OW or OB (BMI >85%): 23% |
| Zhang (5) | 2016 | US | Randomized prospective longitudinal study (Wisconsin CF Neonatal Screening Project)<br>n=107 | • Adult ht sig ↑ in those dx by NBS vs. non-screened<br>• Remained sig after genetic potential adjustment (32 vs 15%; p=0.006) |
| Wainwright (21) | 2015 | Multinational | Two 24-week, randomized, double-blind, placebo-controlled trials of Lum/iva (TRAFFIC and TRASNPORT)<br>n=1,108 ≥12 yo homozygous F508 | • Sig BMI ↑ from baseline in tx groups (pooled data) (tx diff 0.24-0.28 kg/m$^2$ p<0.001)<br>• ■ Not stratified by age |
| Stalvey (22) | 2017 | US | ENVISION (48 wk randomized, placebo-controlled, double-blind trial of ivacaftor)<br>GOAL (24 wk longitudinal obs study of ivacaftor)<br>n=83 aged 6-11 yrs w/≥1 G551D | • Ht sig ↑ from baseline<br>• ■ GOAL (z-score ↑ 0.1 SD p<0.05)<br>• ■ ENVISION (z-score ↑ 0.17 SD from baseline p<0.001; tx diff vs placebo 0.58 SD p<0.05)<br>• Wt sig ↑ from baseline<br>• ■ GOAL (z-score ↑ 0.26 SD p<0.0001)<br>• ■ ENVISION (z-score ↑ 0.35 SD from baseline p<0.001; tx diff vs. placebo 0.8 SD p<0.001)<br>• HV sig ↑<br>• ■ GOAL (↑ of 2.1 cm/yr from pre-baseline to baseline to 3-6 mo p<0.01) |

*(Continued)*

**TABLE 1** | Continued

| Authors | Year | Location | Study Details | Findings |
|---|---|---|---|---|
| | | | | • ■ ENVISION (tx diff vs. placebo 1.08 cm/yr p<0.05)<br>• WV sig ↑<br>• ■ GOAL (↑ of 4.54 kg/yr from pre-baseline to baseline to 6 mo p<0.0001)<br>• ■ ENVISION (tx diff vs. placebo 3.11 kg/yr p<0.001) |
| Taylor-Cousar (23) | 2017 | Multinational | 24 wk randomized, double-blind, placebo-controlled, parallel group trial of Tez/iva (EVOLVE)<br>n= 504 ≥12 yrs homozygous F508 | • No sig diff in BMI ↑ between tx & placebo |
| Ratjen (24) | 2017 | Multinational | 24 wk randomized, double-blind, placebo-controlled trial of Lum/iva<br>n=204 6-11 yrs homozygous F508 | • No sig diff in BMI ↑ between tx & placebo groups |
| Middleton (25) | 2019 | Multinational | 24 wk randomized, double-blind, placebo-controlled trial of Elex/tez/iva<br>n= 403 ≥12 yrs single F508 & minimal function genotype | • Sig BMI ↑ vs. placebo (tx diff 1.04 kg/m$^2$ p<0.001)<br>• Not stratified by age |
| Heijerman (26) | 2019 | Multinational | 4 wk randomized, double-blind, active-controlled trial of Elex/tez/iva<br>n=107 ≥12 yrs homozygous F508 | • Sig ↑ wt (tx diff 1.6 kg p<0.001)<br>• Sig ↑BMI (tx diff 0.6 kg/m$^2$ p<0.0001)<br>• Not stratified by age |
| Owen (27) | 2021 | UK | Cross-sectional study<br>n=37 aged 5-8 yrs | • Ht, BMI, $FEV_1$ within nml<br>• Wt and body comp sig ↓ reference data<br>• ■ 5% FFMI z-score <1 despite optimal BMI |
| CFF Annual Report 2020 (28) | 2020 | US | n=31,411 (13,444 children) | • <2 yo<br>• ■ Wt 0-2 years: 44.5%<br>• ■ Ht 0-2: 31.5%<br>• ■ WFL 0-2 years: 62.8%<br>• 2-19 yo<br>• ■ Wt 2-19 years: 51.9%<br>• ■ Ht 2-19: 38.7%<br>• ■ BMI 2-19 years: 61.4% |
| Marks (29) | 2021 | US | Retrospective case-control study<br>n=3,655 | • Max ht between 2-4 yrs highly correlated w/max adult height (r=0.64) |

*Wt, weight; ht height; abx, antibiotic; PERT, pancreatic enzyme replacement therapy; dx, diagnosis; WV, weight velocity; HV, height velocity; M, male; F, female; pts, patients; diff, difference (s); sig, significantly; yo, year-olds; NBS, newborn screen; yrs, year(s); nml, normal; OW, overweight; OB, obese; tx, treatment; mo, month(s); lum/iva, lumacaftor/ivacaftor; wk, week(s); obs, observational; tez/iva, tezacaftor/ivacaftor; elex/tez/iva, elexacaftor/tezacaftor/ivacaftor; comp, composition; WFL, weight-for-length; max, maximum.*

finding that was attributed to lack of the pubertal growth spurt (**Table 1**). Skeletal maturation was evaluated in 40 of the 50 children and was "retarded" in 25%. Notably, the authors also observed an inverse relationship between severity of respiratory disease and growth (p=0.005 for height; p<0.001 for weight), though the methods used for categorizing respiratory status were not reported (9) (**Table 2**).

## THE 1970'S

By 1974, life expectancy reached 16 years (38) and several investigators began to describe the growth patterns and pubertal maturation of children and adolescents with CF (10–12, 38). Antibiotic agents and treatment intensity improved between the 1960's and 1970's (10), such that standard therapy in the 1970's included use of antimicrobials based on susceptibility and clinical status, aerosol inhalations or nightly mist therapy, postural drainage and chest physiotherapy, vitamin supplementation and PERT (11, 38). In 1979, encapsulated enteric-coated pancreatic enzyme microspheres were introduced. This formulation was designed to reduce gastric acid and pepsin-mediated inactivation, thereby delivering more active enzyme to the duodenum (39) and was shown to be effective in the treatment of pancreatic insufficiency (40).

In 1972, Kreiβl and colleagues evaluated growth parameters in a cohort of 53 patients. Similar to Sproul and Huang, their group identified an association between weight percentiles and disease severity as determined by Shwachman-Kulczycki scores. However, they found no association between disease severity and height (**Table 2**). The median heights in this particular group ranged from the 25$^{th}$ to the 50$^{th}$ centile, with a normal height velocity in all but 7 of the 53 children. As observed in previous studies, weight tended to be more affected than height (**Table 1**). Weight velocity, however, was considered normal in all but 11 of the 53 children (10).

As in the study by Kreiβl, Mitchell-Heggs and colleagues reported a median height at the 25$^{th}$ centile in boys and between the 25-50$^{th}$ centiles in girls. The authors noted that the mean weight percentiles tended to be lower than the mean height percentiles for most (**Table 1**). They also reported that puberty tended to be delayed, but there was no significant relationship between skeletal maturity and disease severity (12).

Results from Berry et al. were similar to those that Sproul and Huang reported in 1964 (9). They too observed an increase in weight following early diagnosis that stabilized 1 SD below the mean from 2 to 8 years of age. This was followed by a gradual decline in height and weight velocity with weight more significantly affected than height (11) (**Table 1**).

**TABLE 2** | Relationship between Growth and Pulmonary Function.

| Author | Year | Location | Study Details | Findings |
|---|---|---|---|---|
| Sproul (9) | 1964 | US | Longitudinal study over ≥ 2 yrs<br>n=50 infancy through adolescence | • Severity of respiratory disease assoc w/ht (p=0.005) & wt (p<0.001) |
| Kreiβl (10) | 1972 | Germany | Longitudinal study over 1-12 yrs<br>n=53 aged 2-13.8 yrs | • Wt assoc with SK score (r=0.53)<br>• ▪ No assoc between SK score & ht |
| Berry (11) | 1975 | US | Case-control study of 'nutritional supplement'<br>n=63 | • Wt & ht pos correlated to clinical score<br>• ▪ Stronger assoc with wt than ht |
| Greco (32) | 1993 | Italy | Longitudinal study 3-14 yrs<br>n=28 aged 4.5 to 17.5 yrs | • Resp and GI events coincide with descending phase of HV and WV |
| Byard (33) | 1994 | US | Longitudinal study<br>n=230 aged 4.75 to 22.25 yrs | • RV/TLC assoc with HV at TO, PHV and PHV +2 yr |
| Nir (15) | 1996 | Denmark | Cross-sectional study<br>n=223 aged 0.75-41 yrs | • Sig assoc between BMI & $FEV_1$ (p<0.0001) |
| Konstan (34) | 2003 | US | Longitudinal study through at least age 6 yrs<br>n=931 | • $FEV_1$ highest in those who maintained wt >10% from 3-6 yrs<br>• $FEV_1$ lowest in those whose wt remained <10% from 3-6 yrs |
| Stallings (35) | 2008 | US | NA | • $FEV_1$ (~≥80%) assoc with BMI ≥50% |
| Sheikh (36) | 2014 | US | Cross-sectional study<br>n=208 aged 5-21 yrs | • LBMI-z & BMI-z pos assoc with $FEV_1$ (not FMI-z)<br>• LBMI-z more strongly assoc with $FEV_1$ vs. BMI-z<br>• ▪ F p<0.0001 vs. p=0.001<br>• ▪ M p<0.0001 for both |
| Hanna (20) | 2015 | US | Cross-sectional study<br>n=226 aged 2-18 yrs | • $FEV_1$ lowest in nutritional failure p<0.0005<br>• ▪ No sig diff in other 4 wt groups including normal wt vs. obese |
| Owen (27) | 2021 | UK | Cross-sectional study<br>n=37 aged 5-8 yrs | • WB FFMI & BMI pos assoc with $FEV_1$<br>• WB FFMI more strongly assoc with $FEV_1$ vs. BMI (p=0.02 vs. p=0.08) |
| Sanders (37) | 2021 | US | Longitudinal study through age 7 yrs<br>n=5,388 | • $FEV_1$ higher in children at 6-7 yo who maintained ht >50% vs. those with ht <50% after adjusting for BMI |

*Assoc, associated; ht, height; wt, weight; SK, Shwachman- Kulczycki score; resp, respiratory; GI, gastrointestinal; HV, height velocity; WV, weight velocity; RV, residual volume; TLC, total lung capacity; TO, take-off; PHV, peak height velocity; yr, year(s); LBMI-z, lean body mass index z-score; pos, positively; FMI-z, fat mass index z-score; F, female; M male; sig significant; diff difference; WB FFMI whole body fat free mass index; yo years old.*

In 1976, Stern et al. reported on a cohort of 95 patients and noted that weight-for-height was generally 'deficient' with weight often under the 3rd centile and height below average. Menarche was noticeably delayed with a mean age of >14 years (38).

Throughout the 1970's, many studies revealed that weight was more significantly affected than height and that puberty and/or skeletal maturity tended to be delayed.

## THE 1980'S

By the early 1980's the mean survival for those with CF increased to 19 years in the United States and 21 years in Canada (13). One notable change to the care of individuals with CF was early detection through newborn screening. In 1985, a comprehensive evaluation of a newborn screening program in Wisconsin began (16). There were also significant changes to nutrition. In the early 1980's, for example, enteric-coated pancreatic enzyme microspheres significantly increased fat absorption over conventional pancreatic enzyme formulations (41). A seminal paper by Corey and colleagues in 1982 demonstrated a significant growth discrepancy between children treated at the CF center in Toronto and those treated in Boston and speculated that these discrepancies were due to differences in PERT dosing and dietary fat intake. Both males and females in Toronto were significantly taller than those in Boston; males in Toronto were also significantly heavier than those treated in Boston, where dietary fat intake was limited (13) (**Table 1**). These findings led to the adoption of the high-calorie, high-fat diets that became fundamental in the care of children with CF.

Soutter et al. reported that most of the children in their cohort had a weight under the 50th centile with a decline in late childhood and pre-adolescence. However, weight and height velocity nearly doubled in those receiving overnight gastrostomy tube feeds, highlighting the importance of caloric (energy) intake. The mean height in their cohort was under the 50th centile (often ranging between the 25 and 50th centiles) (42). These data are similar to those published by Kreiβl and Mitchell-Heggs in the 1970's but reflect a significant improvement from those reported by Sproul in the 1960's (9, 10, 12).

Similar to Soutter et al., Keller and colleagues noted that the mean height in their population was significantly lower than the general population, as were weight and BMI z-scores. However, BMI z-scores in young children were significantly higher in the cohort born in the 1980's and 1990's compared to the cohort born in the 1960's and 1970's, supporting an overall improvement in nutritional status over time (14) (**Table 1**).

Puberty in individuals with CF tended to be delayed in the 1980's with reports of delayed peak height velocity (PHV) (15 years) in boys (43) and delayed menarche (14.5 years) in girls (44). Some authors attributed the delayed puberty to disease severity and nutritional status (44), while others found no such association (45).

Throughout the 1980's, height, weight and BMI in children with CF remained below the general population, though they appeared to be greater than earlier cohorts. The median survival had also increased such that it approximated 30 years by the late 1980's (15).

## THE 1990'S

In the 1990's there was a focus on airway clearance through use of inhaled DNAse to thin the DNA-rich viscous secretions as well as anti-pseudomonal therapy. In a landmark study in 1994, inhaled DNAse (Pulmozyme) improved pulmonary function more than placebo and resulted in fewer pulmonary exacerbations (46). Use of inhaled tobramycin, an anti-pseudomonal therapy, every other month improved pulmonary function, reduced the need for IV antibiotics, and increased weight compared to placebo (47).

The 1990's appear to be a turning point with a general improvement in growth patterns. In fact, the mean height of Swedish children approximated that of the general population (z-score of -0.3 SD) by age 5 y (48). Early identification of CF through newborn screening likely contributed to the improvement in growth observed throughout the decade. In fact, infants diagnosed by newborn screening (mean age 12 weeks) had significantly greater length or height z-scores (-0.2 SD vs. -1.2 SD), weight z-scores (-0.5 SD vs. -1.2 SD) and head circumferences than those identified by symptoms (mean age 72 weeks) (16). Furthermore, those diagnosed by newborn screening were taller and heavier up to 10 years later compared to those diagnosed by symptoms alone (16) (**Table 1**).

Authors from the UK reported mean weight z-scores ranging from -0.25 and -0.5 SD in their population under age 23 (49). Although these were lower than the general population, this reflects a general improvement from data published in the 1970's (11, 38). However, as seen in previous studies (11, 42), these authors also reported a decline in weight z-scores after the first decade of life with BMI z-scores declining from 0 to -0.5 SD to -0.5 to -1 SD (49).

Nir et al. studied a cohort of 223 patients in Denmark after high fat diets and routine anti-pseudomonal therapy became standard. Their group also observed a decline in BMI over time, such that BMI values were lower than the general population by late childhood and early adolescence (**Table 1**). In addition, they identified a strong positive correlation between nutritional status (as indicated by BMI) and $FEV_1$ ($p<0.0001$) (15) (**Table 2**). Greco et al. reported a regular intermittent (pulsatile) pattern in height and weight velocity with most respiratory or GI events coinciding with a descending phase of height and weight velocity (32) (**Table 2**). Similarly, Byard demonstrated a significant correlation between height velocity and pulmonary function (residual volume/total lung capacity) at the onset of the pubertal growth spurt, PHV and 2 years later (33) (**Table 2**).

Lai et al. analyzed data from 13,116 children participating in the National Cystic Fibrosis Patient Registry in 1993 and reported that the median height fell along the 30$^{th}$ centile with weight more significantly affected (20th centile), as observed in previous studies (4, 10–12, 38) (**Table 1**). Malnutrition, as defined by height or weight under the 5$^{th}$ centile was common during infancy. Similar to Nir et al., Lai and colleagues found that malnutrition was also common in adolescence (15). More girls (29%) than boys (19%) were short (height less than the 5$^{th}$ centile) between the ages of 11-14 with the opposite trend noted between the ages of 15-18 (4), potentially related to differences in pubertal timing.

Indeed, Byard reported on 230 children in the US and noted that PHV was lower in magnitude and occurred later than the general population (33). Similarly, in a small study of 17 girls with CF, both age at PHV and age of menarche were significantly later than the general population (50).

Previous studies from the 1960's and 1970's demonstrated an increase in weight upon initiation of appropriate therapy for CF (9, 11), likely due, in part, to the association between growth and disease severity identified by several groups in the 1990's (15, 32, 33).

Despite the overall increase in growth parameters in the 1990's, additional studies continued to report a general decline in weight z-scores after the first decade of life (4, 15, 49), comparable to findings in earlier decades (11, 42).

## THE 2000'S

Airway clearance and anti-pseudomonal therapy remained important therapeutic factors in the 2000's. Similar to inhaled DNAse therapy, inhaled hypertonic saline, used to rehydrate the airway surface and improve mucociliary clearance, reduced pulmonary exacerbations and improved pulmonary function over placebo (51). In addition, oral azithromycin was associated with a reduced risk of pulmonary exacerbations and improved pulmonary function over placebo for those with chronic pseudomonas aeruginosa infections (52).

Studies on newborn screening continued to show a benefit of early detection. The mean height (-0.6 SD) and weight z-scores (-0.23 SD) of those without MI (less likely to be identified early by symptoms alone) approximated those of the general population (17) (**Table 1**). Furthermore, when studied for up to 13 years, the odds ratios for the risk of height or weight under the 10$^{th}$ centile in the non-screened group compared to the screened group were 4.62 and 4.12 respectively (18, 53) (**Table 1**). Data continued to support an association between growth and pulmonary function (34) and, in 2008, evidence-based practice recommendations advocated for individuals aged 2-20 years to maintain a BMI ≥50%, given the association with improved pulmonary function (35) (**Table 2**).

Pulmonary function was also associated with PHV. Specifically, those classified as having severe disease based on a diminished $FEV_1$ had a PHV that was delayed and of lower magnitude than those with milder disease (54).

In a study of 84 Polish children, mean height SD was -0.57 with only 11% of the population having a height z-score more than 2 SD below the mean (55), a stark contrast from the nearly

45% in a notably smaller cohort reported by Shwachman and Kulczycki almost 5 decades earlier (7). The mean BMI z-score in this cohort was -0.77 SD with 33% being classified as malnourished (BMI <10%) and 1 child categorized as obese (55). In the UK, 10.2% of children were categorized as overweight or obese with mean BMI z-score ranging from -0.28 to 0.8 SD in boys and -0.28 to 0.00 SD in girls (19). As observed in earlier studies (15, 49), mean BMI tended to be lower in later childhood and adolescence compared to infancy and early childhood (19) (**Table 1**).

Data published in 2004 demonstrated that height and weight z-scores were not significantly different in a cohort of 15 non-oxygen dependent children with CF compared with their age and sex-matched controls. Furthermore, there were no significant differences in body fat percentage, fat mass, fat-free mass or resting energy expenditure between the two groups (56).

Studies from the 2000's continue to demonstrate an association between growth and pulmonary function. In general, height and weight appear greater than those recorded in previous decades (**Table 2**) with some children meeting criteria for overweight or obesity (19, 55).

## THE 2010'S

Nationwide newborn screening in the United States occurred by 2010 (57). This allowed for early detection and early nutritional intervention for infants with CF. It also permitted an opportunity for aggressive PERT dosing, which was associated with a favorable change in weight-for-age and weight-for-length at age 2 years (58).

In a multi-center longitudinal observational cohort study of 231 infants diagnosed with CF by newborn screening (BONUS study), infants achieved a weight z-score of -0.04 SD by 12 months of age, a significant increase over a cohort who did not undergo screening 20 years earlier (59). Although length z-scores increased over the earlier cohort, they remained low through 12 months of age (**Table 1**). In an additional study, adult height was significantly greater in those diagnosed by newborn screening (50 vs. 29$^{th}$ centile) compared to those diagnosed by symptoms, a finding that remained significant after adjusting for genetic height potential (32 vs. 15$^{th}$ centile) (5) (**Table 1**).

Similarly, VanDevanter reported an increase in mean height z-scores and pulmonary function that occurred in parallel to an increase in the proportion of infants diagnosed by newborn screening between 1994 and 2012 (60).

As with studies in the 2000's (19, 55), Marília da Silva Garrote and colleagues in Brazil categorized nearly 1/3 of their population as malnourished (BMI <10%) and approximately 15% as overweight or obese (defined as BMI >75%). The risk of colonization with pseudomonas aeruginosa was 2.3 times higher in those who were malnourished compared to those who were not (61), a finding that may have contributed to the overall improvement in nutritional metrics and possibly inflammatory status with the initiation of anti-pseudomonal therapy.

These data are in contrast to those presented by Hanna and Weiner, in which only 7% of children in a single United States center were categorized as malnourished (BMI <10%), over half as healthy (BMI 25-85%) and nearly a quarter as overweight or obese (BMI >85%) (20) (**Table 1**).

By the early 2010's, the median childhood BMI reported in the Cystic Fibrosis Foundation Annual Report approached the 50% (28). This decade marked the beginning of more targeted therapies, namely CFTR-modulatory agents (**Table 3**). The first CFTR potentiator, ivacaftor, was FDA approved in 2012 for use in individuals ≥6 years of age with relatively uncommon (gating) mutations, comprising approximately 5% of the CF population (62, 63).

Several studies demonstrated a significant increase in growth and nutritional status in children with at least one G551D mutation treated with ivacaftor. Specifically, ivacaftor treatment for 48 weeks resulted in more weight gain (1.9 kg, $p<0.001$) and a greater increase in BMI z-score (0.45 SD, $p<0.001$) in children aged 6-11 years when compared to placebo (ENVISION) (64).

Stalvey and colleagues performed a *post-hoc* analysis of 83 children ages 6-11 years enrolled in the ENVISION and the GOAL study, a 6-month longitudinal observational study of ivacaftor therapy in those with at least one G551D mutation. Height and weight z-scores increased significantly from baseline in both studies (height z-score increase of 0.1 SD in GOAL and 0.17 SD in ENVISION; weight z-score increase of 0.26 SD in GOAL and 0.35 SD in ENVISION). Height velocity increased significantly in both studies (increase of 2.1 cm/yr between 3-6 months of treatment in GOAL and 1.08 cm/yr greater than placebo in ENVISION). Furthermore, weight velocity was significantly greater in the ivacaftor group compared to the placebo group in ENVISION

**TABLE 3** | CFTR Mutations and Modulator Therapies.

| Mutation Class (more severe to less severe) | Impact on CFTR | CFTR Modulator Therapy | Example of CFTR Modulator |
|---|---|---|---|
| I | Defect in CFTR protein production | | |
| II (example Phe508del) | Ineffective CFTR protein processing → ineffective trafficking to cell surface | Corrector +/- potentiator | Ivacaftor/lumacaftor<br>Tezacaftor/ivacaftor<br>Elexacaftor/tezacaftor/ivacaftor |
| III (example G551D) | Inability of CFTR protein to remain open "gating mutations" | Potentiator | Ivacaftor |
| IV | Ineffective CFTR protein with reduced chloride transport | Potentiator | Ivacaftor |
| V | Decreased CFTR synthesis | | |
| VI | Reduced CFTR stability | | |

(treatment difference of 3.06 kg/yr in boys and 2.81 kg/yr in girls) (22) (**Table 1**).

In a 3-month observational study of 23 patients with at least one gating mutation ranging in age from 5 to 61 years, those treated with ivacaftor had a significant increase in weight, which was positively correlated to the change in $FEV_1$ (p=0.028). Furthermore, there was an increase in fat free mass with a reduction in resting energy expenditure in response to ivacaftor therapy (65).

Ivacaftor therapy was also associated with a significantly greater increase in BMI and BMI z-score in children ≥ 6 years of age with non-G551D gating mutations when compared to placebo (treatment effect 0.7 $kg/m^2$ and 0.28 SD respectively) (66). However, when used to treat individuals ≥ 12 years of age who were homozygous for Phe508del, there were no significant differences in change in weight or BMI between those treated with ivacaftor or placebo (67).

A few years after the FDA approval of ivacaftor, lumacafator/ivacaftor (corrector/potentiator) received FDA approval for individuals ≥12 years of age who were homozygous for the most common mutation in *CFTR*, the Phe508del (**Table 1**) (63). Data pooled from two large 24-week randomized double-blind placebo-controlled trials of lumacaftor/ivacaftor in individuals ≥12 years of age homozygous for Phe508del (TRAFFIC and TRANSPORT) demonstrated a significant increase in BMI in the treatment groups (treatment difference of 0.24-0.28 $kg/m^2$). Unfortunately, these data were not stratified by age, and the specific impact in children was not reported (21) (**Table 1**). When these data were stratified by severity of pulmonary disease, the increase in BMI in the treatment group remained significant across all levels of pulmonary function (68).

In a separate 24-week randomized double-blind, placebo-controlled trial of lumacaftor/ivacaftor in children aged 6-11 years who were homozygous for Phe508del, there was an increase in BMI in both the treatment and placebo groups without a significant treatment difference (24).

Tezacaftor/ivacaftor (corrector/potentiator) was approved in 2018 for individuals homozygous for the Phe508del mutation as well as those with a single Phe508del mutation and one of 26 other mutations (62). In a study of individuals ≥12 years of age homozygous for Phe508del, BMI increased from baseline to 24 weeks in both the tezacaftor/ivacaftor and placebo groups with no significant treatment effect reported (23).

In 2019, elexacaftor/tezacaftor/ivacaftor (next generation corrector/corrector/potentiator) received FDA approval to treat the nearly 90% of individuals harboring at least 1 copy of the Phe508del mutation (62).

In a large multi-center, randomized, double-blind, active controlled trial of elexacaftor/tezacaftor/ivacaftor for individuals aged ≥12 years homozygous for Phe508del, there was a significant increase in mean weight (1.6 kg) and BMI (0.6 kg/m2) compared to those treated with tezacaftor/ivacaftor alone. Unfortunately, these data were not stratified by age and the impact on the pediatric population was not reported (26). The treatment difference in BMI (mean treatment difference of 1.04 $kg/m^2$) was also significant when elexacaftor/tezacaftor/ivacaftor was compared to placebo in individuals aged ≥12 years with a single Phe508del mutation and a minimal function genotype. Again, these data were not stratified by age and the specific impact on the pediatric population could not be assessed (25) (**Table 1**). Additional study is needed to evaluate the impact of modulator therapies, including triple combination therapy, on long-term growth outcomes in children.

## THE 2020'S

The nutritional status of children with CF in the 2020's remains comparable to the general population. In fact, median weight and BMI for children and adolescents aged 2-19 are 51.9% and 61.4% respectively. Likewise, the median weight and weight-for-length during infancy and early childhood are 44.5% and 62.8% respectively (28) (**Table 1**).

Despite overall improvements in nutritional metrics, however, length and height remain below those of the general population (median length 31.5% for ages <24 months; median height 38.7% for ages 2-19 years) (28) (**Table 1**). This continues to be an important area of investigation, as early childhood height is associated with adult height. In fact, Marks et al., found that the maximum height measurements between ages 2 and 4 years are highly correlated with maximum adult height at age 18 or 19 (r=0.64) (29) (**Table 1**). Furthermore, children who maintain a height-for-age over the $50^{th}$ centile have a higher $FEV_1$ at age 6 or 7 than those who have a height-for-age less than the $50^{th}$ centile for at least 1 year. The relationship between height and pulmonary function in this study was not affected by BMI adjustment (37), suggesting that linear growth alone plays an important role in pulmonary function (**Table 2**).

Recent evidence suggests that BMI may not be a reliable indicator of nutritional status in children with CF. Owen et al. reported on body composition and its association with pulmonary function in 37 pre-pubertal children (27). They found that 5% of their population had a fat-free mass index (FFMI) z-score of < -1 [near the value of hidden depletion previously published in adult CF studies (69)] despite optimal BMI z-scores. $FEV_1$ was more strongly associated with whole body FFMI than with BMI (27), supporting the importance of body composition on pulmonary function previously observed in both children and adults (36, 70) (**Table 2**).

## CONCLUSION

Since cystic fibrosis was first described in 1938, there have been many breakthroughs in diagnosis and management. Such developments include the use of antibiotics to treat pulmonary infections, dietary modifications to increase fat and caloric intake, use of pancreatic enzymes, anti-pseudomonal therapies, early identification and intervention through newborn screening, use of mucolytics, and treatment with CFTR modulators, all of which have played a critical role in improving the life expectancy and quality of life for individuals with CF.

Growth in children with CF has historically been poor with weights and heights far below the general population, attributed to malabsorption, reduced caloric intake, increased resting energy expenditure, glucocorticoid exposure and systemic inflammation (71). In addition, historical data support an indirect impact of *CFTR* genotype on nutritional status through the association with pancreatic exocrine function (72). Whether there is a direct effect of CFTR on growth remains unknown.

Previous studies reported a greater impact on weight than height, resulting in BMI z-scores less than 0 SD. Over the last several decades, however, the weight and height z-scores seem to be increasing with some children now meeting criteria for overweight or obesity. While this could reflect an improvement in both weight and height with a more significant improvement in weight relative to height, it appears to be the result of improved weight with minimal increase in height (28, 73). One plausible explanation is a direct effect of CFTR on the GH-IGF-1 axis. However, the exact mechanism remains unclear and additional study is warranted.

While historical data demonstrate a clear association between nutritional status and lung function (34, 74, 75), more recent data support an association between height in early childhood and subsequent pulmonary function regardless of nutritional status (BMI) (37). Therefore, it will be important to identify factors that contribute to early life growth impairment and monitor the impact of highly effective CFTR modulator therapy on linear growth.

Given the minimal increase in height z-scores relative to weight, updated nutritional guidelines will be of great importance in the era of early detection and early treatment with highly effective modulator therapy to optimize growth and pulmonary function while avoiding obesity and its comorbidities. Body composition may provide better insight into nutritional status and its impact on clinical outcomes than BMI alone.

## AUTHOR CONTRIBUTIONS

KM and AR conceived the idea for the topic, both contributed to the outline; KM wrote the first draft and KM and AR contributed to the multiple revisions. All authors contributedto the article and approved the submitted version.

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# Functional hypothalamic amenorrhea: Impact on bone and neuropsychiatric outcomes

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Functional hypothalamic amenorrhea is a state of reversible hypogonadism common in adolescents and young women that can be triggered by energy deficit or emotional stress or a combination of these factors. Energy deficit may be a consequence of (i) reduced caloric intake, as seen in patients with eating disorders, such as anorexia nervosa, or (ii) excessive exercise, when caloric intake is insufficient to meet the needs of energy expenditure. In these conditions of energy deficit, suppression of the hypothalamic secretion of gonadotrophin-releasing hormone (with resulting hypoestrogenism) as well as other changes in hypothalamic-pituitary function may occur as an adaptive response to limited energy availability. Many of these adaptive changes, however, are deleterious to reproductive, skeletal, and neuropsychiatric health. Particularly, normoestrogenemia is critical for normal bone accrual during adolescence, and hypoestrogenemia during this time may lead to deficits in peak bone mass acquisition with longstanding effects on skeletal health. The adolescent years are also a time of neurological changes that impact cognitive function, and anxiety and depression present more frequently during this time. Normal estrogen status is essential for optimal cognitive function (particularly verbal memory and executive function) and may impact emotion and mood. Early recognition of women at high risk of developing hypothalamic amenorrhea and its timely management with a multidisciplinary team are crucial to prevent the severe and long-term effects of this condition.

KEYWORDS

**functional hypothalamic amenorrhea, estrogen deficiency, bone health, anxiety, depression, adolescent**

## Introduction

Functional hypothalamic amenorrhea (FHA) is a condition characterized by irregular or absent menses due to suppression of the hypothalamic–pituitary–ovarian (HPO) axis, and the condition is termed 'functional' because no anatomical or organic disease is identified (1). Abnormalities in gonadotropin-releasing hormone (GnRH) secretion (2, 3) result in hypogonadotropic hypogonadism with impaired luteinizing hormone (LH) pulsatile secretion (4–8), and insufficient LH and follicle-stimulating hormone (FSH) concentrations (5, 9) to maintain full folliculogenesis and therefore ovulatory function, and the condition is also referred to as functional hypogonadotropic hypogonadism. FHA is a common cause of secondary amenorrhea in young premenopausal women and results in severe hypoestrogenism. According to the American Society of Reproductive Medicine, FHA is responsible for 20–35% of secondary amenorrhea (10). It typically occurs in the setting of low body weight, such as anorexia nervosa (AN), excessive exercise (exercise induced or athletic amenorrhea), stress, or a combination of these factors (5). AN is a chronic, relapsing disease defined in the Diagnostic and Statistical Manual -5 (DSM-5) as a state of low body weight in the setting of altered body image and fear of weight gain (11). The Female Athlete Triad (TRIAD) refers to the triad of low energy availability, menstrual dysfunction, and low bone mineral density (BMD) (12–14). Energy deficiency, from either a frank deficit in caloric intake or relative to excessive exercise, leads to hormonal adaptations that aim to optimize energy availability and prioritize this for body functions essential for survival.

Adolescents and young women with FHA typically present with amenorrhea of 6 months' duration or longer (15). However, menstrual status can vary ranging from subclinical menstrual dysfunction (including a shortened luteal phase or anovulatory cycles) to frank oligo-amenorrhea. In adolescents, this condition may be difficult to distinguish from immaturity of the hypothalamic–pituitary–ovarian axis during the early postmenarchal years. However, several reports now indicate that even during the initial postmenarchal years menstrual cycles in adolescents typically are no longer than 45 days, thus irregular or absent menses are concerning (16–18).

The process by which GnRH is suppressed in FHA is multifactorial, as there are many inhibitory and stimulatory neuromodulatory signals that impair GnRH pulsatility. Kisspeptin plays a fundamental role in regulating reproductive function. In the human brain, kisspeptin neurons are found in the hypothalamus, basal ganglia, and periventricular region (19, 20). Kisspeptin has been implicated as the common intermediate signaling factor modulating GnRH activity, acting downstream of leptin and other neuromodulatory systems (21). Studies have demonstrated reduced kisspeptin secretion in conditions of energy deficit in rodents (22). This may be mediated by reductions in levels of hormones such as leptin, insulin, and insulin-like growth factor-1 (IGF-1), and increases in hormones such as ghrelin, cortisol, and adiponectin (23–26).

There may also be a genetic predisposition for the development of FHA. One small study identified heterozygous mutations in the fibroblast growth factor receptor 1 gene *FGFR*, the prokineticin receptor 2 gene *PROKR2*, the hypothalamic gonadotrophin-releasing hormone receptor gene *GNRHR*, and the Kallmann syndrome 1 sequence gene *KAL1* (27) in patients with FHA, suggesting an increased vulnerability to develop hypothalamic amenorrhea. These mutations were not found in healthy controls. However, these findings have not been replicated and more data are necessary to determine whether these findings hold in a larger sample.

The prolonged hypoestrogenemia in FHA has profound effects on many body systems including metabolic, skeletal, neuropsychiatric, and reproductive systems. Estrogen plays an important role in bone health and therefore estrogen deficiency can impact bone mass deleteriously (4, 28). This is particularly important during adolescence, a critical period for bone mass accrual, and lack of estrogen during this time can lead to decreased BMD and increased fracture risk, both immediate and in the long-term. Another area of concern is the impact of prolonged hypogonadism and changes in hormones such as cortisol, leptin, and peptide YY (PYY) on neurocognitive status, emotion, and mood (29–32), thus posing additional challenges at an age when emotional lability is already common. The adolescent years are very important for optimal development of neuropsychiatric function and normoestrogenemia may be essential in this context.

Many young women are not aware of these long-term effects of FHA; thus, it is important to identify these women early, and address with them the importance of adequate energy availability and resumption of menses. Treatment includes correction of the energy deficit state to improve GnRH pulsatility and restore normal functioning of the HPO axis.

## Impact of conditions associated with functional hypothalamic hypogonadism on bone

In situations of prolonged hypoestrogenism, as seen in states of functional hypothalamic (or hypogonadotropic) hypogonadism, changes are noted in areal BMD (aBMD), bone microarchitecture and strength estimates, associated with an increased risk of fractures in these individuals.

### Anorexia nervosa and bone

Bone health has been extensively reviewed among adolescents with AN. Compared to controls, adolescent and

young adult women with AN have low aBMD, which is driven independently by both low body weight (percent expected body weight for height, EBW-Ht, ≤ 80 or 90%) and amenorrhea (33), and alterations in hormones such as IGF-1, the gonadal steroids, cortisol, leptin, insulin, adiponectin, PYY and oxytocin. Older studies indicate that as many as 67% of these individuals may have an aBMD Z-score < -2 (34) while more recent findings (given earlier diagnosis of AN) report that ~ 52% have an aBMD Z-score < -1 at one or more sites, with trabecular bone (spine) being commonly affected (35). Of particular concern, in AN, bone accrual is stalled during adolescence, a vital period that determines long term bone health and fracture risk (36, 37). BMD improves with weight gain and menstrual recovery. However, it is uncertain whether full catch-up occurs and whether this is sufficient to ensure optimal long-term bone health (37).

Furthermore, studies using high-resolution peripheral quantitative computed tomography (HRpQCT) and microfinite element analysis (μFEA) (Figure 1) have demonstrated changes in cortical and trabecular volumetric BMD (vBMD), bone geometry and microarchitecture, and bone strength estimates in AN. At the distal radius (a non-weight bearing site), compared to controls, adolescents and young women with AN have lower total and trabecular vBMD, increased cortical porosity, lower cortical area and thickness, increased trabecular area and separation, and lower strength estimates (stiffness and failure load) (38). At the distal tibia (a weight-bearing site) adolescents and young adult women with AN, compared with controls, have lower total and cortical vBMD, increased cortical porosity, lower cortical area and thickness, lower trabecular number and lower stiffness and failure load (39). Another study showed, lower trabecular bone volume fraction (BV/TV) and trabecular thickness and higher trabecular separation in girls with AN using flat panel volume computed tomography (CT) when compared with normal-weight controls (40). IGF-1, leptin and androgen levels predict bone microarchitecture in adult women with AN, with lower levels appearing to have deleterious effects (41). Marrow adipose tissue (MAT) measured by 1H-magnetic resonance spectroscopy (1H-MRS) is higher in AN than controls, indicating increased differentiation of the common mesenchymal progenitor stem cell in marrow along the adipocyte rather than the osteoblast lineage, and higher marrow adipose tissue is related to lower bone strength estimates in young women with this condition (39).

These bone changes translate to higher fracture risk in adolescents and adult women with AN compared with controls that persists over time (42–44). One study reported no difference across groups in the site of fracture (upper extremity, lower extremity or non-extremity); the AN group just had many more fractures across all sites than the control group (44).

## Exercise induced amenorrhea and bone

Similarly, in oligomenorrheic athletes, bone density, bone microarchitecture and strength are altered, a consequence of both low energy availability (from insufficient caloric intake and increased metabolic demands) and hypoestrogenism, as well as alterations in hormones such as IGF-1, other gonadal steroids, cortisol, and other metabolically regulated hormones. Oligoamenorrheic athletes have lower spine, hip and whole body aBMD than eumenorrheic athletes (45, 46). As in AN, there is concern that catch-up may be insufficient even after

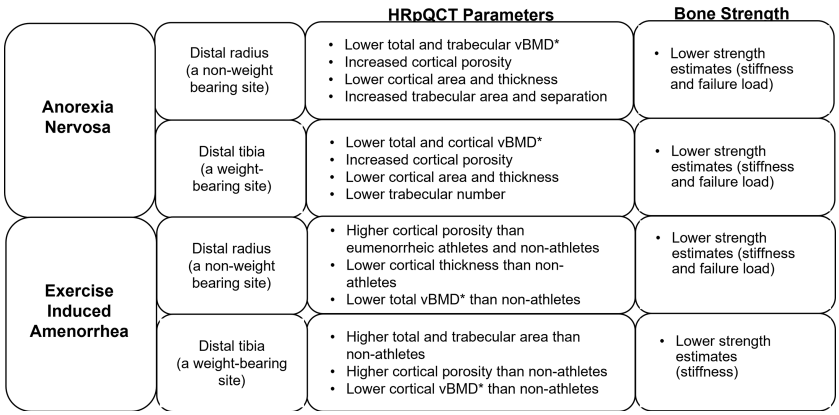


FIGURE 1
Impact of functional hypothalamic amenorrhea on bone parameters as assessed by high-resolution peripheral quantitative computed tomography (HRpQCT) and microfinite element analysis (μFEA). HRpQCT was used to assess volumetric bone mineral density (vBMD)*, bone geometry and structure, and μFEA to assess bone strength estimates at the distal radius and tibia.

menses resume given the narrow window for optimizing bone accrual during and after puberty (47, 48).

However, using only BMD to assess bone health may be insufficient (14). In oligomenorrheic athletes, HRpQCT has been used to study changes in bone geometry and microarchitecture, and findings differ at non-weight bearing compared with weight bearing sites (Figure 1). At non-weight bearing sites such the distal radius, oligomenorrheic athletes have higher cortical porosity and lower strength estimates than eumenorrheic athletes, and higher cortical porosity with lower cortical thickness and total vBMD than non-athletes (46). At weight bearing sites such as the tibia, oligomenorrheic athletes have higher total and trabecular area, higher cortical porosity and lower cortical vBMD than non-athletes, and lower strength estimates than both eumenorrheic athletes and non-athletes (46, 49). Further, in one study, trabecular number was lower and separation higher in oligomenorrheic athletes compared to eumenorrheic athletes and non-athletes at the tibia (50). More recently, a study assessed the effects of energy deficiency (defined as energy intake below 45 kcal/kg fat-free mass/day) in long-distance triathletes without hypoestrogenism compared to non-athletes and found that several bone parameters (total and trabecular area, trabecular vBMD and trabecular microstructure) were better in athletes than non-athletes (consistent with the adaptive effects of bone loading), but inferior in athletes with low energy availability compared to those with adequate energy availability (51).

Fractures, particularly stress fractures, are more prevalent among oligo-amenorrheic athletes than eumenorrheic athletes and non-athletes (46). A history of two or more fractures in oligo-amenorrheic athletes has been associated with lower spine and whole-body BMD Z-scores, lower radial cross-sectional area, trabecular vBMD and strength estimates, and lower tibial strength estimates compared to oligoamenorrheic athletes with less than 2 fractures (46). Thus, the protective mechanism bestowed by mechanical loading on athletes is deficient in a state of hypoestrogenism.

## Anorexia nervosa versus female athlete triad

Bone health and fracture risk are affected differently in adolescents and young adult women with AN and oligo-amenorrheic athletes (52). In AN, whole body less head (WBLH) and hip aBMD Z-scores, and several measures at the weight-bearing tibia (total vBMD, cortical area and thickness, trabecular number, and estimated strength) were lower than in oligo-amenorrheic athletes and controls in one study (52). In contrast, both AN and oligo-amenorrheic athletes had lower spine aBMD Z-scores, lower radius total, cortical and trabecular vBMD, cortical area, cortical thickness, and estimated strength, and lower tibial cortical vBMD than controls (52). Although fracture risk was higher in women with AN and oligo-amenorrheic athletes, the fracture type varied with nonstress fractures being more common in AN and stress fractures being more common in oligo-amenorrheic athletes.

# Determinants of bone health in functional hypothalamic hypogonadism

Determinants of bone outcome in these patients include changes in body composition, alterations in the HPO axis, growth homone-IGF-1 axis, hypothalamic-pituitary-adrenal axis, and appetite regulating and other hormones, consequent to the low energy availability state (Figure 2).

## Body composition

In addition to the lower body mass index (BMI), changes in body composition in the setting of energy deficiency impact bone health. Overall, restrictive caloric intake in AN leads to lower fat and lean mass and lower resting energy expenditure (53). A compensatory increase in cortisol concentrations occurs in energy deficient states such as AN and exercise induced amenorrhea, and in AN, higher cortisol concentrations are associated with lower extremity lean mass (54). Lower lean mass is an independent predictor of BMD at almost every site, consistent with the pull of muscle on bone being osteogenic (37). With weight regain, increases in lean mass are associated with increases in BMD (36). Further, marrow adipose tissue (known to reduce biomechanical strength) is higher in AN than controls and in oligo-amenorrheic athletes than in eumenorrheic athletes and is associated with lower BMD and lower strength estimates (55, 56). In contrast, cold-activated brown adipose tissue is lower in AN. Some believe that brown adipose tissue plays a role in the differentiation of a common marrow progenitor mesenchymal stem cell preferentially into osteoblasts instead of adipocytes (57). This would explain a direct association of lower brown adipose tissue with lower BMD and an inverse relationship with levels of pre-adipocyte factor-1 (pref-1), a hormone that inhibits differentiation of this mesenchymal stem cell along the osteoblast pathway (57).

## Hypothalamic-pituitary-ovarian axis

The gonadal steroids, including estrogen, testosterone and dehydroepiandrosterone (DHEA) (an ovarian and adrenal androgen and estrogen precursor), have important effects on bone (28).The estrogens (estradiol and estrone) inhibit osteoclastic bone resorption by increasing osteoprotegerin and decreasing receptor activator of nuclear factor kappa-B ligand

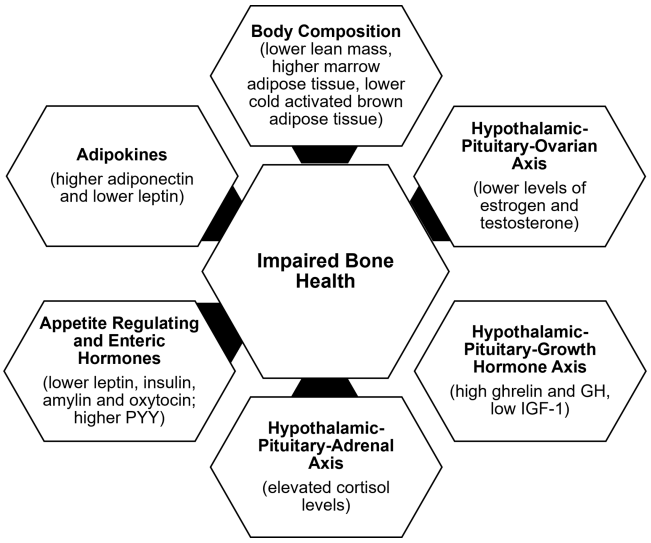


FIGURE 2
Factors contributing to impaired bone health in functional hypothalamic amenorrhera.

(RANKL) secretion by osteoblasts (28). They may also increase bone formation by inhibiting secretion of sclerostin and pref-1, both of which otherwise inhibit osteoblast differentiation. Effects of testosterone on bone are mediated *via* its aromatization to estrogen; however, it also has direct osteoanabolic and anti-resorptive effects. DHEA is weakly bone anabolic, and also anti-resorptive through its aromatization to estrogen. Levels of estrogen and testosterone are lower in AN and in oligo-amenorrheic athletes compared with controls (36, 50). The duration of amenorrhea, consistent with the duration of hypogonadism, and menarchal age predict the extent of bone health impairment.

## Hypothalamic-pituitary-growth hormone axis

Energy deficiency results in a state of growth hormone (GH) resistance with elevated GH concentrations and low IGF-1 levels in AN vs. controls (58), and in amenorrheic athletes vs. non-athletes (45), consistent with a hepatic resistance to GH, likely mediated by a downregulation of the GH receptor in end organs (as indicated by lower levels of GH binding protein) (59) and elevated fibroblast growth factor (FGF)-21 concentrations (60). This state of GH resistance is commonly seen in conditions of undernutrition. In athletes, lower IGF-1 levels have been associated with higher intensity of training (61, 62). GH stimulates osteoblast precursors and mature osteoblasts both directly and indirectly through the action of IGF-1 (63). While higher GH concentrations are associated with higher levels of bone turnover markers in healthy normal-weight controls, this association is not evident in AN (despite higher GH concentrations), indicative of a resistance to GH at the level of bone (in addition to the liver) (58). A study using supraphysiologic doses of recombinant human GH demonstrated a decrease in fat mass in women with AN (consistent with its IGF-1 independent lipolytic effects) without a corresponding increase in IGF-1 concentrations or concentrations of P1NP (a bone formation marker), further corroborating a hepatic and skeletal resistance to GH in AN (64). Further, low IGF-1 concentrations are associated with low bone density in conditions of functional hypothalamic hypogonadism (36, 45), and administration of replacement doses of recombinant human IGF-1 (rhIGF-1) has been associated with an increase in levels of bone formation markers in adolescents and adults with AN (65, 66).

## Hypothalamic- pituitary-adrenal axis

One of the neuroendocrine adaptations of FHA includes overactivity of the hypothalamic–pituitary–adrenal axis (HPA), with increased secretion of corticotropin-releasing hormone (CRH), adrenocorticotropin hormone (ACTH), cortisol, and endogenous opioids (67–71). Higher cortisol levels have also been found in the cerebrospinal fluid of women with FHA compared to eumenorrheic women (7, 72). There is a tight link between activation of the HPA axis and reduction in GnRH

drive in those with FHA (5, 70, 73, 74), such that an increase in CRH suppresses GnRH pulsatility (7). Further, cortisol inhibits kisspeptin release (75, 76), and its elevation contributes to the cascade of impairment in GnRH release, with higher cortisol concentrations being associated with lower secretion of LH (70). One study has shown that in AN, adolescents with the lowest BMI, fat mass, fasting glucose and insulin levels (thus with the lowest energy availability) have the highest cortisol concentrations, suggesting that the increase in cortisol levels is an adaptative mechanism, likely to maintain euglycemia in a state of low availability given its gluconeogenic effects (71).

These relatively high cortisol levels may have immediate and long-term effects on bone health in patients with FHA. The deleterious effects of hypercortisolemia on bone are mediated by many different mechanisms including reduced osteoblastic activity, increased osteoclastic activity, inhibition of intestinal calcium absorption, impaired renal handling of calcium, and reduced secretion of GH and IGF-1 (77). Higher cortisol levels predict lower percent extremity lean mass (54) and lower bone density in FHA (30, 71). As previously discussed, lean body mass is an important determinant of bone density (35, 78). High cortisol levels in women with AN are inversely correlated with markers of bone turnover and may contribute to low BMD through suppression of bone formation (71, 79).

### Insulin, enteric peptides and adipokines

Appetite regulating hormones (such as leptin, insulin, PYY, and oxytocin, which are anorexigenic, and ghrelin, which is orexigenic) as well as adipokines, such as adiponectin, are modulators of energy availability and play a critical role in the regulation of hypothalamic dysfunction and in bone metabolism in FHA (80, 81). Leptin and insulin stimulate kisspeptin release, while ghrelin and adiponectin inhibit its release (25, 75, 76, 82, 83). Similarly, PYY can modulate reproductive function (84, 85). Women with FHA have lower leptin (6, 8, 9, 86–90), insulin (9, 86, 87, 91), and oxytocin levels (92), and higher ghrelin (8, 90, 93–95), PYY (94, 96, 97), and adiponectin (91, 98) levels than controls. In FHA, many of these hormonal alterations have been associated with suppression of the HPO axis (25, 80, 81, 99, 100).

All these hormonal alterations contribute to hypogonadism in FHA and consequently to low bone mass. In addition, these hormones have direct effects on bone. Leptin is osteoanabolic and antiresorptive (101–103), and insulin, amylin and ghrelin also have bone anabolic effects. Lower levels of leptin, insulin and amylin correlate with lower BMD and impaired bone microstructure in those with AN (41, 91, 104). Ghrelin levels correlate with bone endpoints in healthy normal-weight controls, but not in girls with AN, consistent with a ghrelin resistant state (105). PYY inhibits osteoblastic activity (106), and in AN, high PYY levels are associated with lower BMD in adults, and with lower levels of bone turnover markers in adolescents (96, 107). Similarly, higher PYY levels are associated with lower levels of bone formation markers and lower BMD in adolescent athletes and non-athletes (97). Oxytocin is now known to be bone anabolic, and lower oxytocin concentrations in AN have been associated with lower bone density (108). Adiponectin receptors are expressed on osteoblasts and osteoclasts (109, 110), and high levels of adiponectin are deleterious to bone. High adiponectin levels are associated with low BMD in healthy adults (111, 112) and in girls with AN (91).

## Treatment strategies

The first line of management of FHA is lifestyle intervention, aimed at normalization of HPO axis function and resumption of menses. The approach should be multidisciplinary and include involvement of a physician to coordinate care (preferably an eating disorder specialist), a dietician, and a psychologist or psychiatrist (particularly when there is a co-existing eating disorder), with engagement of the parents or other family members and the athletic trainer or coach (for hyperexercisers). The condition is generally reversible and resolves after restoration of energy balance and resolution of underlying emotional stress. While targeting the triggers of FHA, such as disordered eating, excessive exercise or emotional stress is the first approach, convincing patients to change long-standing behavior can be challenging.

A dietary evaluation and consequent counseling are important to optimize caloric intake (including healthy fat), and micronutrients such as calcium and vitamin D. Energy availability should meet established weight goals and other clinical criteria for athletes to continue exercising, and these athletes may need to modify their training and competition regimen if such goals are not met (46, 113). Consistent with this, sports consensus groups, including the Female Athlete Triad Coalition and International Olympic Committee, recommend that athletes with FHA undergo screening for various components of the Triad and meet certain energy availability requirements to be permitted to continue to exercise (12, 13). In a small study, three out of four amenorrheic athletes resumed menses after a 20-week program that included nutritional supplementation and one rest day per week (114).

Similarly, psychological support for treating stress and enhancing behavioral change is critical (14). Behavioral modifications can result in a reduction in cortisol levels (115) and resumption of ovarian function in some women with FHA (116). One study showed that 71% of patients recovered over a period of 7-9 years and predictive factors of recovery included lower serum cortisol concentrations and higher basal BMI (117).

There has been some debate over whether a critical increase in weight is required to resume menstrual cycles. Based on one study, a recommendation is that goal weight should be at least 2 kg higher than the weight at which point menses were lost (118). This longitudinal study involving adolescents with AN

also showed that menstruation resumed at a mean body weight that was 91.6 ± 9.1% of ideal body weight and that it could take 6-12 months or longer of being at a 'healthy' weight before menses resumed (118). Another study suggested that about 50% of women are expected to resume their menstrual cycle when they are at or above a BMI of 19 kg/m$^2$ with ≥ 23% body fat (119). Overall, the recommendations are to regain any recent weight loss, to aim for a body weight that is at least 2 kg greater than at which menses were lost and a body weight that corresponds to greater than 90% of median BMI for age (or greater than 18.5 kg/m$^2$ for adults). However, there is significant variability in the set point for menstrual recovery from one individual to another, thus establishing goal weight can be challenging. Further, if a woman continues to be amenorrheic despite being at a healthy weight for a prolonged period, it is important to consider other causes of amenorrhea such as persistent emotional stress or conditions such as polycystic ovarian syndrome.

In women, particularly athletes, who are at a 'healthy' weight and yet have FHA, low caloric intake may still be a contributor to the amenorrheic state, these women often have lower fat mass than eumenorrheic women, consistent with a state of energy deficit. In such women, careful assessment of caloric intake and expenditure may be necessary to demonstrate the state of low energy availability. Individualized meal plans are often helpful in such instances, and it is important to emphasize optimizing caloric intake before and after periods of intense exercise. Recommendations from the Female Athlete Triad Coalition include increasing calories through intake of food such as nuts, dried fruit, energy bars and drinks, avocado and fatty fish (12).

## Management of low bone density

The management of low bone density in FHA is summarized in Figure 3. Table 1 summarizes interventional studies addressing bone outcomes in FHA.

The most important strategy to improve bone density in adolescent and adult women with FHA is normalization of menstrual function and recovery of weight (for those who are undernourished, underweight or have had recent weight loss). It is important to supplement vitamin D to maintain 25(OH) vitamin D levels above 30 ng/ml (135) and to recommend adequate calcium intake (1000-1500 mg daily). Clinicians should obtain a baseline BMD measurement by dual-energy X-ray absorptiometry (DXA) for any adolescent or woman with 6 or more months of amenorrhea, and even earlier in patients with a history of severe nutritional deficiency, other energy deficit state, and/or skeletal fragility (136).

Although estrogen deficiency is an important determinant of low BMD in FHA, many studies have shown lack of a protective effect of combined oral contraceptives (COCs) on bone (120–122, 137). A possible reason for the lack of efficacy of oral estrogen in increasing BMD is the suppression of IGF-1, a key osteoanabolic hormone (particularly during the adolescent years), by COCs because of hepatic first pass metabolism (123, 138). In contrast, transdermal 17-β estradiol, administered in replacement doses, is not IGF-1 suppressive (138, 139), and randomized clinical trials (47, 124) over 12 or 18 months have demonstrated that transdermal 17-β estradiol replacement is effective in increasing spine and hip BMD Z-scores in oligo-amenorrheic athletes and adolescents with AN, although catch-

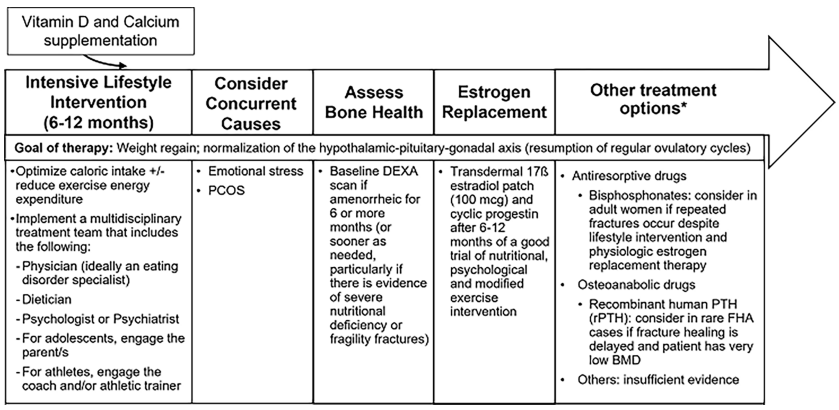


FIGURE 3
Management of Functional Hypothalamic Amenorrhea. HPO, Hypothalamic-pituitary-ovarian axis; PCOS, Polycystic ovarian syndrome; DEXA, Dual-energy X-ray absorptiometry; rPTH, Recombinant parathyroid hormone. *Treatment is similar in both adult and adolescent women except for "other treatment options" which at this time, only apply to adults.

TABLE 1 Interventional studies in the management of functional hypothalamic amenorrhea.

| Study | Population | Intervention | Outcomes |
|---|---|---|---|
| ***Oral estrogen-progestin therapy*** | | | |
| Klibanski A, et al. The Journal of Clinical Endocrinology & Metabolism. 1995 (120). | 48 amenorrheic women with AN mean age 23.7 years | Estrogen and progestin replacement (n=22) vs. no replacement (n=26) for a mean of 1.5 years | Intervention (estrogen and progestin) group had no significant change in BMD compared to the group that received no hormone replacement therapy. On post-hoc analysis, very low-weight women with <70% ideal body weight treated with estrogen and progestin had a 4.0% increase in mean BMD while the group that did not receive replacement treatment had a 20.1% decrease in BMD. |
| Warren MP, et al. Fertility and Sterility. 2003 (121). | 55 dancers (n=24) with amenorrhea mean age 22 years | Individuals with amenorrhea received conjugated equine estrogen, 0.625 mg, vs. placebo for 25 days, with medroxyprogesterone acetate 10 mg, for 10 days of every month over 2 years. Both groups were compared to eumenorrheic controls. | There was no significant difference in BMD at the lumbar spine, wrist and foot in the treated or placebo group compared to the eumenorrheic dancers.BMD increased but did not normalize in 5 individuals who resumed menses. |
| Strokosch GR, et al. Journal of Adolescent Health. 2006 (122). | 112 adolescent girls with AN or eating disorder not otherwise specified 11-17 years old | A combined oral contraceptive (COC) (norgestimate 180-250 μg and ethinyl estradiol 35 mcg) vs. placebo for 13 cycles of 28-day cycles | Significant increase in BMD at the lumbosacral spine and hip in the intervention group at week 6.<br>No statistically significant effect on lumbosacral spine or hip BMD at the end of 13 cycles. |
| ***Transdermal estradiol administration with cyclic progestin*** | | | |
| Ho KKY, Weissberger AJ. Journal of Bone and Mineral Research. 2009 (123) | 14 postmenopausal women (two groups n=7 each) | Oral (20 μg/day of ethinyl estradiol) vs. transdermal (100 μg/day of 17β-estradiol) estrogen over 2 months | Transdermal estrogen significantly increased IGF-1, procollagen III, procollagen I, osteocalcin and fasting urinary hydroyproline to creatinine ratio (UOHPr/Cr) while oral estrogen administration led to suppression of these biochemical endpoints. There was a significant association between IGF-1 elevation and changes in procollagen III, procollagen I, osteocalcin and UOHPr/Cr. |
| Misra M, et al. Journal of Bone and Mineral Research. 2011 (124). | 110 girls with AN and 40 normal-weight controls 12-18 years | Girls with AN: Those with a bone age of ≥15 years (n=96) received 100 μg of 17β-estradiol + cyclic medroxyprogesterone acetate vs. placebo, while those with a bone age of <15 years (n=14) received incremental low-dose oral ethinyl-estradiol vs. placebo for 18 months. 40 normal-weight controls were followed without intervention for the study duration | Spine and hip BMD and BMD Z-scores improved in the group with AN that received physiologic estrogen replacement to approximate bone accrual rates observed in controls. |
| Ackerman KE, et al. British Journal of Sports Medicine 2019 (47) | 121 normal-weight athletes with amenorrhea 14-25 years | 100 μg 17β-estradiol transdermal patch + cyclic 200 mg oral micronized progesterone vs. 30 μg ethinyl estradiol and 0.15 mg desogestrel pill vs. no estrogen or progesterone over 12 months | Spine and femoral neck BMD Z-scores significantly increased in the estrogen patch vs. the estrogen pill and no estrogen groups. Hip BMD Z-scores increased in the estrogen patch vs. the oral pill group |
| Singhal V, et al. The Journal of Clinical Endocrinology & Metabolism. 2019 (48). | 73 oligo-amenorrheic females 14-25 years | 100 μg 17β-estradiol transdermal patch twice weekly + cyclic 200 mg oral micronized progesterone vs. 30 μg ethinyl estradiol and 0.15 mg desogestrel pill vs. no estrogen or progesterone over 12 months | N-terminal propeptide of type 1 procollagen (P1NP), a marker of bone formation, decreased most in the pill group and this was associated with lower IGF-1 levels in the pill group vs. the other two groups, which did not have a decrease in IGF-1 levels; the pill group also demonstrated a marked increase in SHBG compared to the other two groups.<br>The transdermal group had the greatest increases in estradiol levels and demonstrated decreases in sclerostin, preadipocyte factor-1 (Pref-1) and brain derived neurotrophic factor (BDNF). The increase in estradiol was directly related to increases in BMD. |
| Ackerman KE, et al. Journal of Bone and Mineral Research 2020 (125) | 75 oligo-amenorrheic females 14-25 years | 100 μg 17β-estradiol transdermal patch twice weekly + cyclic 200 mg oral micronized progesterone vs. 30 μg ethinyl estradiol and 0.15 mg desogestrel pill vs. no estrogen or progesterone over 12 months | Total and trabecular volumetric BMD, bone geometry and structural parameters improved in the estrogen patch vs. the estrogen pill group, particularly at the distal tibia. |
| ***Other treatment options*** | | | |

*(Continued)*

TABLE 1 Continued

| Study | Population | Intervention | Outcomes |
|---|---|---|---|
| Grinspoon S, et al. J Clin Endocrinol Metab. 2002 (126). | 60 adult women with AN and osteopenia in their 3rd decade of life | rhIGF-1 30 μg/kg SC BID + COC 35mcg ethinyl estradiol and 0.4 mg norethindrone vs. rhIGF-1 alone vs. COC alone vs. neither over 9 months | Combined therapy (rhIGF-1 plus COC) group had the greatest increase in BMD compared to the group that received neither. |
| Welt CK, *et al.* New England Journal of Medicine. 2004 (80). | 14 women with FHA, 19-38 years | Metreleptin, recombinant human leptin, (n=8) vs. placebo (n=6) over 3 months | Treated group showed improved reproduction function and many resumed menses.<br>Free triiodothyronine, free thyroxine, IGF-1, IGF binding protein 3, and bone formation markers (bone specific alkaline phosphatase and osteocalcin) increased in treated group.<br>Treated group had a decrease in body weight and fat mass. |
| Golden NH, *et al.* The Journal of Clinical Endocrinology & Metabolism. 2005 (127). | 32 adolescent girls with AN, mean age 16.9 years | Alendronate 10 mg daily vs. placebo over 1 year | Small but significant increase in BMD at the femoral neck, but not at the spine, in the alendronate vs. placebo groups after controlling for body weight changes over the study duration. Body weight was the best predictor of improved BMD |
| Chou SH, *et al.* Proceedings of the National Academy of Sciences. 2011 (128) | 20 adult women with FHA 19-34 years | Metreleptin (n=11) vs. placebo (n=9) over 36 weeks | Improved menstrual function but no difference in spine, hip, radius or total BMD in the treated vs. placebo groups (though bone mineral content increased in the metreleptin group).<br>Significant increase in a bone formation marker (osteocalcin).<br>However, the treated group had a decrease in body fat. |
| Miller KK, et al. J Clin Endocrinol Metab. 2011 (129). | 77 adult women with AN, in their 3rd decade of life | Risedronate 35 mg weekly vs. low-dose transdermal testosterone vs. combination therapy vs. placebo over 12 months | Risedronate lead to increased posteroanterior spine, lateral spine and hip BMD compared to placebo.<br>Testosterone administration was not associated with increased BMD but was associated with increased lean body mass |
| Divasta AD, et al. Metabolism. 2012 (130). | 80 young women with AN 13-27 years | DHEA (50 mg daily) + COC (20 μg ethinyl estradiol + 0.1 mg levonorgestrel) (n=43) vs. placebo (n=37) over 18 months | DHEA + COC group had maintenance of spine and whole-body areal BMD Z-scores while placebo group had a decrease in areal BMD Z-scores over the study duration |
| Fazeli PK, et al. The Journal of Clinical Endocrinology & Metabolism. 2014 (131). | 32 women with AN, mean age 47 years | Recombinant PTH (n=21) vs. placebo (n=11) over 6 months | Spine BMD improved in the recombinant PTH group compared to the placebo group |
| Divasta AD, et al. J Adolesc Health. 2019 (132). | 70 adolescent girls with AN 11-17 years | Oral micronized DHEA (50 mg daily) + COC (20 μg ethinyl estradiol + 0.1 mg levonorgestrel) (n=35) vs. placebo (n=35) over 12 months | Reduction in BMD Z-scores in girls with open epiphysis and no change in girls with at least one closed epiphysis with combination therapy compared to placebo<br>No effect of treatment on pQCT parameters at the tibia |
| Haines MS, et al. J Bone Miner Res. 2021 (133). | 90 women with AN and low areal BMD Z-scores, 19-35 years | Sequential therapy with rhIGF-1 over 6 months followed by risedronate for 6 months (n=33) vs. 12 months risedronate (n=33) vs. placebo (n=16) | rhIGF-1+risedronate therapy was associated with greater spine areal and volumetric BMD than the placebo group and greater spine areal BMD than the other groups. |
| Haverinen A, et al. The Journal of Clinical Endocrinology & Metabolism. 2022 (134). | 59 healthy women, 18-35 years | Estradiol valerate 2 mg + dienogest 2-3 mg (n=20) vs. ethinyl estradiol 30 μg + dienogest 2 mg (n=20) vs. dienogest 2mg (n=19) over 9 weeks | Lower levels of SHBG, and less pronounced FSH suppression leading to higher estradiol levels in the estradiol valerate vs. the ethinyl estradiol and dienogest groups. |

Anorexia nervosa (AN); combined oral contraceptive (COC); dual energy x-ray absorptiometry (DEXA); functional hypothalamic amenorrhea (FHA); Bone mineral density (BMD); insulin-like growth factor (IGF); parathyroid hormone (PTH); Recombinant human IGF-1 (rhIGF-1); dehydroepiandrosterone (DHEA); peripheral quantitative computed tomography (pQCT).

up is incomplete. This lack of complete catch-up is likely due to residual alterations that persist in other hormones that may impact bone that are not fixed by estrogen replacement. Importantly, the impact of estrogen replacement on fracture risk in women with FHA remains unclear. The Endocrine Society guidelines suggest short-term use of transdermal 17-β estradiol with cyclic oral progestin in adolescents and women with FHA who do not resume menses after a reasonable trial of nutritional, psychological, and/or modified exercise intervention (136).

The route of estrogen administration may have an impact on bone that extends beyond effects on IGF-1. A study that examined the impact of route of estrogen administration in oligo-amenorrheic athletes showed that transdermal estradiol replacement was associated with an increase in estradiol levels (associated with increases in bone density), and a decrease in factors that inhibit osteoblastic activity such as sclerostin, Pref-1, and brain-derived neurotrophic factor (BDNF). Further, while COCs led to a significant increase in sex hormone binding globulin levels with a decrease in levels of bioavailable gonadal steroids, this effect was not observed in the transdermal estrogen group (139). All these mechanisms may contribute to the efficacy of transdermal estrogen (but not COCs) in improving bone outcomes. A study examining effects of estradiol valerate versus ethinyl estradiol in oral contraceptive pills with the same progestin found a less pronounced FSH suppression in the estradiol valerate group, leading to higher estradiol levels and suggesting more positive effects of natural estradiol on bone mass (134).

Adolescent girls and adult women with AN and amenorrheic athletes have lower levels of testosterone than control groups. However, one study of transdermal testosterone given in replacement doses vs. placebo in adult women with AN was not associated with increases in BMD, despite an initial increase in bone formation markers (129).

Few studies have evaluated the use of anti-resorptive medications such as bisphosphonates and denosumab in FHA. One randomized controlled study of risedronate vs. placebo in adult women with AN reported small but significant increases in BMD (2-3%) at the spine and hip (129), while another study of alendronate vs. placebo in adolescents with AN reported a small increase at the femoral neck (but not at the spine) (127). When considering these drugs as a therapeutic strategy (particularly in women who have repeated fractures despite lifestyle intervention, optimization of calcium and vitamin D status, and estrogen replacement), caution needs to be exercised during the reproductive years given concerns regarding their long half-life. Data for denosumab are not available at this time in women with FHA.

Similar to the anti-resorptives, few studies have examined the impact of osteoanabolic drugs [such as teriparatide, recombinant PTH (rPTH), abaloparatide, romosozumab, recombinant leptin (metreleptin), rhIGF-1, and DHEA] on bone outcomes in FHA. A 6-month study of teriparatide vs. placebo in older pre-menopausal women with AN reported improvements in spine BMD (131); however, studies over a longer duration are currently lacking. There are also no studies that have reported on the impact of abaloparatide or romosozumab on bone outcomes in FHA.

Although a small 3-month study of metreleptin vs. placebo in adult women with FHA demonstrated improvement in menstrual function and increases in levels of IGF-1 and markers of bone formation with metreleptin, the medication led to subjective reductions in appetite and a significant decrease in body weight and fat mass (80). A subsequent small 9-month study similarly showed improved menstrual function and an increase in bone mineral content at the lumbar spine following metreleptin treatment. However, the group that received this drug had a significant decrease in body fat despite careful dose titration to prevent weight loss, an undesirable side effect in individuals with FHA (128).

Recombinant human IGF-1 given with a COC was demonstrated to increase spine and hip BMD in adult women with AN in a 9-month RCT in which the women were randomized to receive the combination regimen, rhIGF-1 alone, COC alone or neither (126), suggesting that administering rhIGF-1 in replacement doses may mitigate the IGF-1 suppressive effects of a COC. However, in a 12-month randomized controlled trial in adolescents with AN in which all received transdermal 17-β estradiol (given in replacement doses with cyclic oral progestin) with half being randomized to receive replacement doses of rhIGF-1 and half randomized to placebo, adding rhIGF-1 to transdermal 17-β estradiol did not lead to a further improvement in bone outcomes (140). In contrast, in a study in adults with AN, sequential therapy with rhIGF-1 for 6 months followed by risedronate for 6 months (vs. 12 months of risedronate or double placebo) led to greater increases in spine aBMD and vBMD than in the double placebo group, and greater increases in lateral spine aBMD than in both other groups (133).

Finally, one 18-month study demonstrated that a combination regimen of DHEA (50 mg daily) with a COC (vs. double placebo) led to a maintenance of BMD Z-scores at multiple sites compared to a decrease in these measures in the placebo group in young women with AN 13-27 years old (130). However, a subsequent study of this combination regimen vs. placebo in younger girls with AN 11-17 years old reported a reduction in spine and whole-body BMD Z-scores with the combination regimen in the younger girls with open epiphyses, and no change in these BMD measures in older girls with open epiphyses (132).

Based on these and other studies, current guidelines caution against using denosumab, metreleptin and androgens to improve bone outcomes in FHA. In rare adult FHA cases, the guidelines suggest short-term use of teriparatide as an option in patients with delayed fracture healing and very low BMD (136). Given that recent studies have demonstrated that bisphosphonates improve bone

outcomes in adults with AN (133) this may be a consideration in those women who continue to have fractures despite attention to caloric intake, a reduction in exercise activity, and optimization of vitamin D, calcium, and estrogen status.

## Neuropsychiatric outcomes

Adequate estrogen status is essential for optimal cognitive function and may also impact mood and emotion (141, 142).

**Cognitive Function:** Hypoestrogenism has deleterious effects on verbal memory and executive function (specifically cognitive flexibility) in oligo-amenorrheic athletes compared to eumenorrheic ones and/or non-athletes (143). Other studies have also demonstrated cognitive dysfunction in adolescent and adult women with FHA (144, 145), with an improvement in these measures with menstrual resumption or estrogen administration (144). Importantly, estrogen replacement as the transdermal 17-β estradiol patch with cyclic oral progesterone given for a 6-month period improved both verbal memory and cognitive flexibility in oligo-amenorrheic athletes compared to a no-estrogen group (with a COC group demonstrating intermediate effects) (143).

**Emotion and Mood:** Women with FHA have significantly higher depression and anxiety scores compared to healthy controls (146). Studies reported that depression and anxiety are common in women with FHA, suggesting a role for estrogen in mediating these effects (146–149). Another study reported that administration of transdermal estradiol reduced trait anxiety in girls with AN and prevented the increase in state anxiety observed with weight gain over time than in those who received placebo (150).

**Eating Behaviors and Attitudes:** Women with FHA exhibit more dysfunctional attitudes such as perfectionistic behavior and extra attention to peoples' judgments and have great difficulty coping with daily stress in comparison with eumenorrheic women (151, 152). Additionally, women with FHA report greater internal feelings of insecurity, inadequacy, and lack of control over their lives (146). One study in athletes and non-athletes reported greater cognitive restraint, drive for thinness, feelings of ineffectiveness and greater interoceptive awareness in oligo-amenorrheic athletes compared to eumenorrheic athletes and non-athletes (153). A subsequent study showed a significant improvement in drive for thinness and body dissatisfaction scores, and a reduction in uncontrolled eating after 12 months of treatment with transdermal estradiol with cyclic progesterone (154) (not observed in those who received COCs). Further, transdermal estradiol replacement in adolescent girls with AN has been demonstrated to prevent the increase in body dissatisfaction that occurs with weight gain over time in those who remain hypoestrogenic (150).

**Hormonal Correlates of Neuropsychiatric Outcomes:** Estrogen has an influence on many areas of the brain including to the hypothalamus, cerebellum, nigrostriatal and mesolimbic system, amygdala, hippocampus, cerebral cortex, and brainstem (155). Estrogen also modulates many neurotransmitters including serotonin, acetylcholine, dopamine, and norepinephrine (156). Although hypoestrogenemia plays a major role in the neurocognitive impairment in FHA, hypercortisolemia due to HPA dysregulation and fluctuations in neuropeptides and neurotransmitters can work synergistically to promote the neuropsychiatric disturbances in this condition. One study showed that amenorrheic women present greater increases in heart rate, systolic and diastolic blood pressure, and serum cortisol levels in response to neuropsychological stress exposure than eumenorrheic women (151). In another study of 21 healthy controls, 18 amenorrheic women with AN, and 13 normal-weight women with FHA, cortisol levels showed a strong correlation with anxiety and depressive symptoms (30). Lower levels of gonadal hormones, oxytocin, and leptin, and higher levels of cortisol and PYY have been implicated in eating disorder psychopathology and symptoms of anxiety and depression in AN (31, 32, 108, 157).

The relationship between psychological stress and FHA is bidirectional, as stress can trigger the suppression of the HPO axis and, conversely, low levels of estrogen greatly impact the neuropsychological status, thus creating a vicious cycle. Therefore, psychological support is essential to break the cycle. The Endocrine Society Clinical Practice Guidelines (136) suggest psychological treatment such as cognitive behavioral therapy (CBT) to improve the ability to cope with psychological stressors. In a study, eight women with FHA were randomized to CBT and eight to observation for 20 weeks. Among women who received CBT, most (six of eight) achieved ovulatory recovery compared to only one of eight in the observation group (116). In another study, CBT lowered cortisol levels, and increased leptin and TSH levels in women with FHA (115). The long-term impact of CBT in amenorrheic women needs to be studied.

## Conclusion

FHA from AN, low energy availability in athletes or chronic stress is a frequent cause of oligo-amenorrhea in young women and can go undiagnosed for long periods of time. FHA results from disruption of the HPO axis consequent to other endocrine changes and possibly a genetic predisposition, with an impact on reproductive, neuropsychiatric and skeletal health (summarized in **Figure 4**). Early recognition of patients at risk of developing FHA is very important due to the long-term consequences of low energy availability on the reproductive system, and the impact of low energy availability and hypoestrogenism on bone and neurocognitive outcomes, particularly during the critical adolescent and young adult years when skeletal and neurological systems are maturing. Treatment aims to

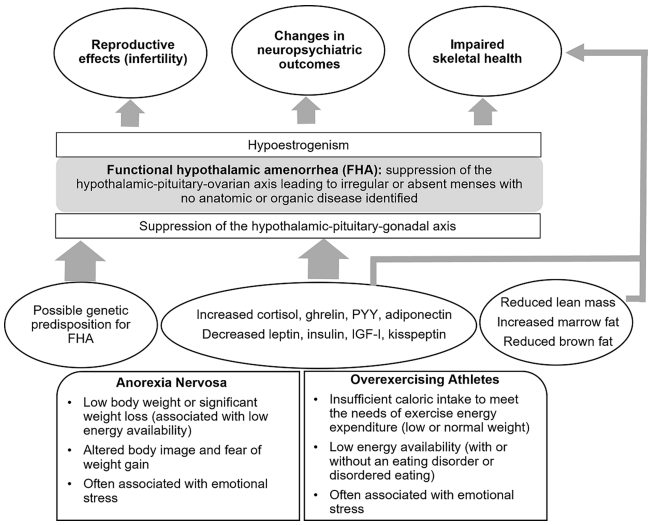


FIGURE 4
Anorexia Nervosa and the Female Athlete Triad.

optimize energy availability with restoration of gonadal function and generally requires a multidisciplinary team. Transdermal estrogen therapy is a proven useful tool in those women who do not respond to nutritional, psychological, and/or modified exercise intervention, and has beneficial effects on bone accrual, as well as neuropsychiatric outcomes.

## Author contributions

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

## 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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# Serum α-KL, a potential early marker of diabetes complications in youth with T1D, is regulated by miRNA 192

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Despite the wealth of information on biomarkers of diabetes complications in adults with type 1 diabetes, data in the pediatric population is limited. Diabetic nephropathy (DN), the leading cause of mortality in type 1 diabetes T1D), could be potentially missed in youth, as albuminuria, the current "gold" standard, may be transient and may not reflect permanent renal impairment. Soluble alpha KL has emerged as a potential marker of early diabetic nephropathy. Seventy-nine pediatric patients with type 1 diabetes meeting ISPAD criteria for nephropathy screening were consecutively recruited (90% Caucasian, 51% male, mean age 16.1 ± 3.1 years, duration of T1D 7.2 ± 3.9 years, 2-year average HbA1c 8.0 ± 1.3%, and serum and urine samples were collected for analysis. Serum Klotho (KL) and circulating miRNA levels of select miRNA involved in the pathogenesis of DN were estimated. KL had a strong inverse correlation with diabetes duration and HbA1c, two important risk factors in the development of diabetes complications. Serum miR-192 were negatively associated with KL among children with prolonged duration of diabetes (≥12 years) after adjustment for age and sex. In cell culture, overexpression of miR-192 significantly downregulated KL mRNA and protein levels, and reduced KL levels in the media. miR-192 mimic reduced luciferase activity in a reporter containing the KL 3' UTR (60% compared to controls, p<0.01), and the inhibitor rescued it. Deletion of a potential binding site for miR-192 in the KL 3'UTR completely abolished the effect of miR-192 in the reporter assay, suggesting that KL is a direct target gene of miR-192. Overexpression of miR-192 significantly increased oxidative stress (MDA) and expression of inflammatory and senescence markers IL-6 and p16. Inhibition of miR-192 significantly reduced levels of MDA, IL-6 and p16. In summary, we demonstrate an increase in miR-192 and a decrease in KL levels in children with prolonged duration of T1D. We demonstrate a novel role for miR-192 in directly regulating KL levels, and through that, senescence and oxidative stress, key pathological

processes in the development of DN. miR-192 and/or KL levels are altered with severity and duration of diabetes and could serve as early biomarkers for DN.

KEYWORDS

type 1 diabetes mellitus, Klotho, miR-192, diabetic nephropathy, biomarker

## Introduction

Diabetic nephropathy (DN) is a significant complication of type 1 and type 2 diabetes. DN is characterized by persistent albuminuria and a progressive decline in renal function. It is reported that the prevalence of DN is between 20% to 50% among those living with diabetes (1), and clinical studies have shown that DN is the most frequent cause of end-stage kidney disease (ESKD) in most countries (1). In the U.S., DN accounted for nearly 50% of all patients (more than 58,000 people) with ESKD (United States Renal Data System, 2018, NIH, NIDDK). The annual prevalence of DN in the pediatric population has also been increasing significantly, with one reported study citing an increase from 1.16% to 3.44% from 2002-2013 (2).

Alpha-Klotho (KL), an obligate co-receptor molecule for fibroblast growth factor (FGF) 23 function, was originally identified as an anti-aging protein (3). KL was found to be expressed in multiple tissues, with highest expression in the kidneys (4–6). Mice lacking KL display multiple aging-like phenotypes including vascular calcification and osteoporosis and died prematurely at around two to three months of age (4), and overexpression of the KL gene extends life span in mice (7). Clinical and basic research have provided evidence that KL is involved in many human diseases including cardiovascular disease, osteoporosis, cancers, and acute and chronic kidney diseases (8). The effects of KL are mediated through regulation of biological processes and signaling pathways including oxidative stress, inflammation, and fibrosis (3). Renal expression of KL as well as circulating KL levels are severely decreased in patients with chronic kidney disease (9) and in experimental animal models of kidney disease (10–13) including in diabetic db/db mice. Most of the published evidence is from adults with diabetes and kidney disease, while studies examining circulating KL levels in children with type 1 diabetes (T1D) are very limited. In a cross-sectional single center study from Poland, sKL was lower in children with T1D than in the controls and correlated with HbA1C, but not duration of diabetes (14).

Even though a correlation between KL and DN has been reported, the regulation of KL levels in DN is still unclear. miRNAs could be potential candidates as they have been shown to play a critical role in DN. Expression of miRNA-192 is increased in glomeruli isolated from both type 1 and type 2 diabetes animal models compared to their nondiabetic controls (15). Importantly, inhibition of miR-192 or miR-192 knockout results in decreased proteinuria and renal fibrosis in streptozotocin-induced diabetic mouse models (16–18). Urinary miR-192 has been correlated with albuminuria in a cohort of patients with T1D (19).

We hypothesized that KL levels will be affected by diabetes duration and glycemic control in children with Type 1 diabetes, and KL levels could be regulated by miRNAs, specifically miR-192. We studied the relationship of circulating KL levels to patient and metabolic characteristics in children with T1D, and the potential role of miR-192 in regulating the levels of KL.

## Research design and methods

Seventy-nine consecutive pediatric patients (age:10.9-23.9 years) with T1D (duration: 2.1-17.6 years) who met International Society of Pediatric and Adolescent Diabetes (ISPAD) screening criteria for nephropathy screening (≥ 10 years of age and ≥ 2 years of duration) were recruited (20). Serum sample was obtained along with random urine collection at recruitment for measurement of albumin/creatinine ratio (ACR). Demographic, anthropometric, and laboratory data was obtained from the institution's EMR. The study was approved by the Institutional review board at University of Pittsburgh (Protocol Number: PRO15100286).

### Serum KL level

Serum α-KL level was determined using ELISA kit from IBL (Cat# 27998, Immuno-Biological Labs, Japan) according to the manufacturer's instructions. The coefficient of variability (%CV) is 2.7-6.5, and the lower detection limit was 6.15 pg/mL.

### Serum miRNA expression

Total RNA was extracted from human serum samples using miRNeasy Serum/Plasma Kit (Qiagen, Germantown, MD), and cDNA was synthesized using miScript II RT kit (Qiagen,

Germantown, MD) according to the manufacturer's instruction. The QuantiTect SYBR Green PCR master mix and miScript Primer assays for miR-192, miR-126, miR21, miR-29a and miR-210 (Qiagen, Germantown, MD) were used to perform miRNA real time quantitative PCR. Relative mRNA levels were calculated by 2−ΔΔCt and normalized to miR-210.

## Cell culture and transfections

Human kidney cell line (HK2) was purchased from ATCC and maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) supplied with 10% FBS and 1X Penicillin-Streptomycin. Medium was changed every other day. For miRNA transfection, reverse transfection was used. Briefly, miR-192 mimic (Life Technologies) or negative control miRNA (Life Technologies) were diluted in 50uL of opti-MEM in a final concentration of 100nM and 1.25ul of Lipofectamine RNAiMax was added and mixed in 24-well plate. After 20 min,1 X $10^5$ cells were seeded into the plated. Cells were harvested 72-h later for further analysis. For plasmid transfection, HK-2 cells were plated in 24-well plate for overnight. Plasmids were transfected into the cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

## Dual reporter assay

The full length KL3'UTR in luciferase reporter construct (kl-wt-luc) was a gift from Dr. Gwen King (Creighton University). The miR-192 binding site deletion construct (kl-mut-luc) was cloned using Q5® Site-Directed Mutagenesis Kit Protocol kit (New England Biolabs, Ipswich, MA). The kl-wt-luc or kl-mut-luc construct was co-transfected into HK2 cells with renilla luciferase control (Rluc). The cells were then transfected with either control miRNA or miRNA-192 mimic, or miRNA-192 mimic + inhibitor for 48 hours. The miR-192 inhibitor is a pre-synthesized anti-miR™ miRNA Inhibitor (ThermoFisher. Ambion). Anti-miR™ miRNA Inhibitors are chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous miRNA molecules. The cells were harvested in lysis buffer and the luciferase activity was measured using Dual-Luciferase® Reporter Assay kit (Promega, Madison, WI).

## Oxidative stress assay

Oxidative stress was assessed in human serum or cell lysate using Thiobarbituric Acid Reactive Substances kit (Cat# 10009055, Cayman Chemical, Ann Arbor, Michigan) according to the manufacturer's instruction.

## RNA extraction and real time PCR

Total RNA was extracted from the cells using RNeasy purification kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. First strand cDNA was synthesized from 1 μg total RNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out using TaqMan assays in a 20 μl reaction mixture (Bio-Rad) containing 0.1 μl first strand cDNA and 1X probe and primers mix (Bio-Rad). Probes were purchased from Life Technologies Relative mRNA levels were calculated by 2−ΔΔCt and normalized to β-actin.

## Western blot

Cells were homogenized in RIPA buffer (50 mmol/l Tris [pH 7.4], 150 mmol/l NaCl, 1% Triton X-100, 0.5% SDS) containing proteinase inhibitors (Roche, Indianapolis, IN, USA) and phosphatase inhibitors. Denatured total protein (30 μg) was resolved on SDS-PAGE, then transferred to PVDF membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (154 mmol/l NaCl) with Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated with primary antibodies (GAPDH (control) and KL; diluted in 5% BSA in TBS-T) overnight at 4°C. The membranes were then washed three times with TBS-T and incubated with horse-radish peroxidase (HRP)-conjugated secondary antibodies (diluted in 5% non-fat dry milk in TBS-T) for an additional 1 h at room temperature. Images were taken after adding SuperSignal West Dura extended duration substrate (Thermo Fisher Scientific) to the membrane. Antibodies were pre-validated by molecular mass using positive control samples.

## Statistical analysis

Descriptive statistics are presented as frequencies with percentages, means with standard deviation, or medians with interquartile range. Kruskal-Wallis and Wilcoxon Rank Sum tests assessed between group difference for continuous variables. Correlations between KL and other variables was assessed using Spearman rank order correlation analysis. Partial correlations were also computed adjusting for age. Additionally, Chi-square tests, t-tests, were used to assess difference between groups for categorical and continuous variables, respectively. Linear or logistic regression models were used to adjust for age, sex, and relevant covariates. miRNA levels were not normally distributed and thus were log10-transformed for analysis. The relationship between miR-192 and diabetes duration was assessed using locally weighted scatterplot smoothing (LOWESS) regression. Because there was a pronounced inflection point at ~12 years of

disease duration, subsequent analyses were stratified by duration <12 vs >12 years. All statistical tests were two-tailed with *P* values of ≤0.05 considered to be statistically significant.

# Results

A total of 79 subjects with type 1 diabetes meeting ISPAD criteria for nephropathy screening were consecutively recruited. Background characteristics are summarized in Table 1. Most subjects were white (90%), half were male (51%), mean ± SD age of 16.1 ± 3.1 (range: 10.2-23.9), age at onset of 8.9 ± 3.8 (range: 1.1-17.2), and duration of T1D of 7.2 ± 3.9 (range: 2-18) years. Body mass index (BMI) percentile (median [IQR]) was 73.5 [45.7-88] and BMI standard deviation score (SDS) (median [IQR]): 0.64 [-0.01-1.17]. In terms of laboratory parameters, HbA1c at the time of the evaluation was 8.3 ± 1.5% (range: 7.3-9), 2-year average HbA1c 8.0 ± 1.3% (range: 7.3-8.5), mean eGFR 99.1 ± 19.9 ml/kg/1.73m$^2$, median [IQR] albumin/creatinine ratio was 9.8 [5.5, 21.1] and serum soluble α-KL 1204 [871, 1537] pg/ml. There were 13 subjects that had abnormal ACR at the study visit (ACR ≥ 30 mg/gr). Of these, 5 had a prior ACR checked with 3 having an abnormal result (5, 11 and 18 months prior to the study visit). One had been prescribed lisinopril with very poor compliance documented in the medical record. All 13 subjects were included in the analysis.

TABLE 1 Characteristics of the patient cohort.

| Variables | |
|---|---|
| Age (years) | 16.1 ± 3.1 |
| Age at onset (years) | 8.9 ± 3.8 |
| Diabetes duration (years) | 7.2 ± 3.9 |
| Female/Male (%) | 49/51 |
| Race (White/Black/Hispanic) (%) | 90/9/1 |
| BMI percentile | 73.5 [45.7-88] |
| BMI SDS | 0.64 [-0.01-1.17] |
| HbA1c at time of evaluation (%) | 8.3 ± 1.5 |
| HbA1c average of 2 years including time of evaluation (%) | 8.0 ± 1.3 |
| Albumin/Creatinine (ACR) (mg/g) | 9.8 [5.5, 21.1] |
| eGFR (ml/kg/1.73 m$^2$) | 99.1 ± 19.9 |
| Serum soluble α-KL (pg/ml) | 1204 [871, 1537] |

Data are presented as mean ± SD except for BMI percentile and BMI SDS, ACR and KL (median [25$^{th}$, 75$^{th}$]).
BMI, Body mass index.
SDS, Standard deviation score.

## Serum α-KL is associated with glycemic control and diabetes duration in a pediatric population with T1D

KL was significantly correlated with HbA1c at time of evaluation, average HbA1c over 2 years, and diabetes duration (Figure 1; Table 2). This remained significant even when adjusted for age. KL did not correlate with ACR. There was no

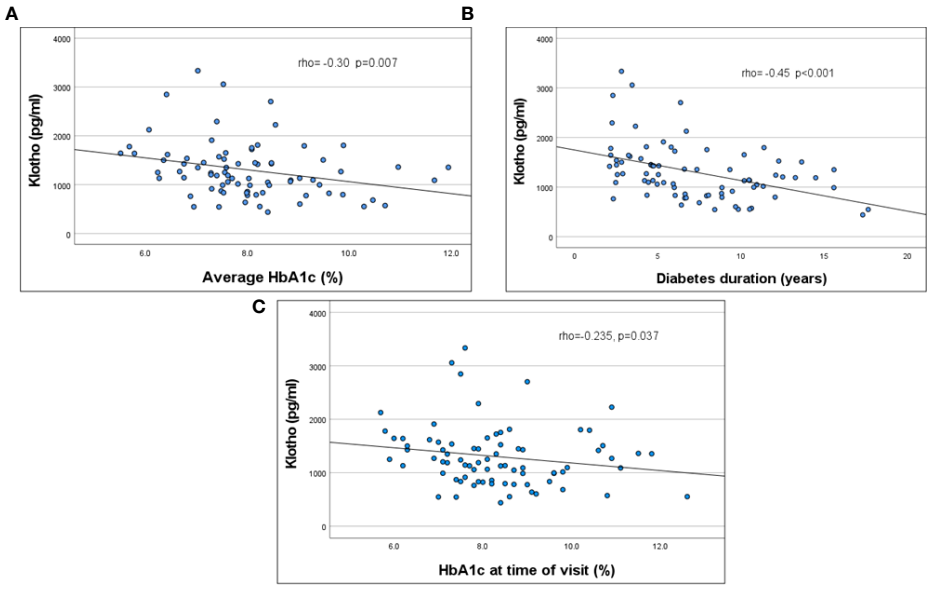


FIGURE 1
Correlation of serum KL levels to HbA1C (2 years average, **(A)** p =0.007), HbA1C at the time of evaluation (**B**, p=0.037) and duration of diabetes (**C**, p<0.001).

TABLE 2 Correlations between KL and other independent variables.

| Variables | R | p-value | N |
|---|---|---|---|
| Age (years) | 0.03 | 0.79 | 79 |
| Diabetes Duration (years) | -0.453 | **<0.001** | 79 |
| A1C at the time of evaluation | -0.235 | **0.037** | 79 |
| Average HbA1c (last 2 years) | -0.301 | **0.007** | 79 |
| Average SBP (%ile) | 0.005 | 0.97 | 72 |
| Average DBP (%ile) | -0.077 | 0.52 | 72 |
| BMI (%ile) | -0.59 | 0.62 | 72 |
| ACR (mg/g) | 0.016 | 0.89 | 75 |
| Triglycerides (mg/dl) | -0.116 | 0.31 | 79 |
| Total Cholesterol (mg/dl) | -0.108 | 0.34 | 79 |
| HLD- Cholesterol (mg/dl) | -0.100 | 0.38 | 79 |
| LDL-Cholesterol (mg/dl) | -0.061 | 0.59 | 79 |

Bold indicates a significant difference.

difference in KL levels by quartiles of ACR or between those with normal (ACR < 30 mg/gr) (n=62) vs abnormal (ACR ≥ 30 mg/gr) (n=13) ACR (1165 [868-1503]vs 1355 [912-1715] pg/ml, p=0.5). There was no significant correlation between KL and age, eGFR, SBP %ile and DBP %ile (Table 2).

## Serum miRNA 192 levels negatively correlate with serum KL in children with longer duration of diabetes

In the study population above, serum expression level of multiple renal function related miRNAs, including miR-192, miR-126, miR-21, miR-29a and miR-210 were estimated (15, 17, 21–23). Circulating miR-192 levels tended to increase with age (p=0.051), but did not correlate with HbA1C, duration of diabetes, BMI percentile, BMI z score, systolic BP, diastolic BP, Total cholesterol, HDL, LDL or VLDL levels. miR -192 levels were relatively stable among subjects of shorter diabetes duration, with a clear inflection point at 12 years of diabetes, after which miR-192 levels increased with time (Figure 2A). Moreover, miR-192 levels negatively correlated with KL levels in children with prolonged duration of diabetes (>12 years) (Figure 2B). After adjustment for age and sex, KL decreased -246.8 pg/ml (95% CI -412.0 to -81.6) per each 1-unit increase in miR-192 relative ratio (n=11, p=0.01, model R2 = 0.65) (Figure 2C). There was no relationship between miR-192 and HbA1C (data not shown). Levels of miR-126, miR21, miR-29a and miR-210 did not correlate with HbA1C, duration of diabetes, BMI percentile. BMI z score, systolic BP, diastolic BP, Total cholesterol, HDL, LDL, VLDL levels or KL levels in our population.

## Overexpression of miR-192 in HK2 cells inhibits the expression of KL

To investigate whether miR-192 regulates the expression of KL, we transfected miR-192 mimic into HK2 cells for 72 hours. We found that the expression level of KL is significantly

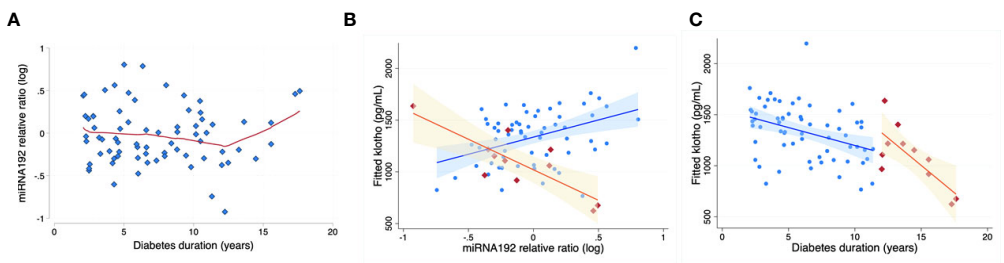


FIGURE 2
miR-192 levels and KL levels with diabetes duration. miR-192 levels are relatively stable with disease <12 years duration. There is an "inflection point" at ~12 years, with miR-192 levels increasing after that with increased duration of diabetes **(A)**. Red line is based on LOWESS (locally weighted scatterplot smoothing) regression. Correspondingly, in a predictive model using miR-192 level and adjusted for age and sex **(B)**, KL levels decrease more steeply after 12 years of duration of diabetes (orange segment; shaded area represents 95% confidence bands). Blue segment demonstrates the relationship in children with diabetes duration of less than 12 years. **(C)** shows significant inverse relationship between miRNA-192 and KL levels with prolonged duration of diabetes (>12 years of diabetes; orange, $r^2 = 0.89$, p=0.03).

suppressed in HK2 cells by miR-192 mimic transfection (Figure 3A). We also found that the protein level of KL is markedly inhibited by miR-192 mimic compared to control (scrambled miRNA) transfection (Figure 3B). In addition, the soluble KL levels in the media, measured using ELISA assay, is significantly decreased in cells transfected with miR-192 mimic (Figure 3C). This demonstrated that miR-192 regulates KL expression at both mRNA and protein levels.

## miR-192 inhibitor mitigates the effects of overexpression of miR-192 in HK2 cells on oxidative stress, inflammation, and senescence

KL plays a critical role in oxidative stress, inflammation, and senescence. Since miR-192 mimic transfection resulted in a dramatic reduction in KL levels, we examined oxidative stress using the TBARS assay for MDA in HK2 cells transfected with miR-192 mimic. We found that transfection of miR-192 mimics causes a 2-fold increase in oxidative stress in HK2 cells (Figure 4A). We also examined the gene expression levels of IL-6 and p16, markers of inflammation and a senescence, and we found that IL-6 and p16 are both significantly upregulated in cells transfected with miR-192 mimics (Figure 4B, C). The co-transfection of miR-192 mimic and the inhibitor mitigated the effects of miR-192 on oxidative stress, inflammation, and senescence, and significantly decreased the levels of MDA, IL-6 and p16 levels (Figures 4A–C). These experiments demonstrate that miR-192 affects oxidative stress, inflammation, and senescence in HK2 cells.

## KL is a potential target of miR-192

miRNAs largely regulate gene expression through directly binding to the 3'UTR of target genes and inducing degradation of mRNAs or decreased mRNA translation. Since miR-192 overexpression suppresses the mRNA levels of KL, we speculate that KL is a miR-192 target gene in kidney cells. We scanned the 3'UTR sequence of KL mRNA and found a potential binding site for miR-192 in the 3'UTR of KL mRNA (Figure 5A). To further examine whether miRNA-192 directly regulates KL expression, we co-transfected miR-192 mimic or the combination of mimic and inhibitor together with the kl-wt-luc or kl-mut-luc reporter constructs. We then performed the dual luciferase reporter assay and found that miR-192 mimic inhibits wt KL 3'UTR luciferase activity, and that the inhibitor abolishes the effects of the mimic (Figure 5B). Furthermore, both mimic and inhibitor lost their effects on the kl-mut-luc 3'UTR reporter construct (Figure 5C), suggesting that miRNA-192 regulates KL expression specifically through binding to the novel miR-192 binding site in the KL 3'UTR.

# Discussion

This is the first study to date to examine levels of KL, miR-192 and their relationship with established biochemical and clinical markers of diabetes and its complications in youth with type 1 diabetes. Our results show that circulating KL levels are negatively correlated to HbA1c and diabetes duration after adjusting for age. Serum levels of miR-192 are negatively associated with circulating KL levels in children with prolonged duration of diabetes, suggesting a regulatory role of miR-192 in the expression of soluble KL. This was confirmed by *in vitro* experiments that demonstrated the role of miR-192 in regulating KL expression and levels. We show that miR-192 is an upstream regulator of KL, and induces markers of oxidative stress, inflammation, and senescence *in vitro*. Finally, we demonstrate that KL is a direct target gene of miR-192.

Despite the wealth of information on biomarkers of diabetes complications in adults with T1D, data in the pediatric population with T1D are limited. DN, one of the most serious chronic complications of T1D and the leading cause of mortality (24), could be potentially missed in children, as albuminuria (the current non-invasive "gold" standard for screening, monitoring,

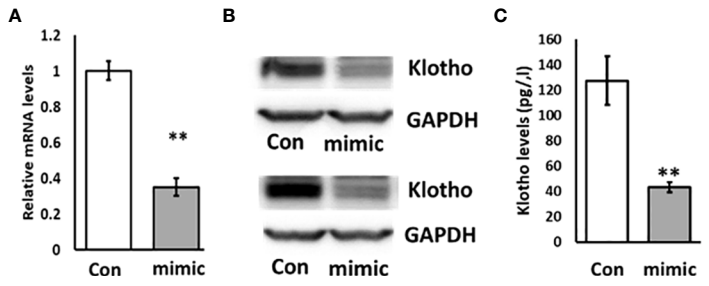


FIGURE 3
Overexpression of miR-192 inhibits the expression of KL in HK2 cells. miR-192 mimic or scrambled miRNA were transfected into HK2 cells for 72 hours and mRNA **(A)**, protein **(B)** and soluble KL in the media **(C)** were detected by real time PCR, immunoblotting and ELISA. **p < 0.01.

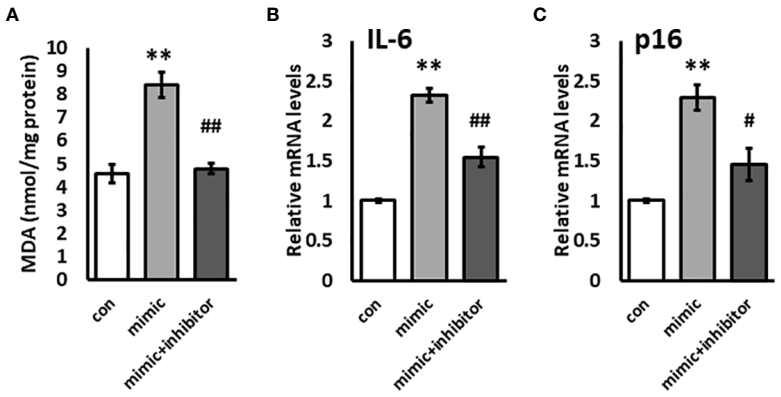


FIGURE 4
Overexpression of miR-192 induces oxidative stress and inflammation in HK2 cells. miR-192 mimic, mimic + inhibitor, or scrambled miRNA were transfected into HK2 cells for 72 hours and MDA levels in the cell lysate **(A)**, and mRNA levels of IL-6 **(B)** and p16 **(C)** were measured. **p<0.01, #p<0.05, ##p<0.01.

and predicting progression of DN in adolescents) may be temporary and may not necessarily reflect permanent renal impairment (25). It is thought that renal hyperfiltration may be the earliest hemodynamic abnormality seen in patients with diabetes and it has been linked with an increased risk of DN with rapid decrease in glomerular filtration rate (GFR) (26). Moreover, several lines of evidence suggest that advanced lesions in both glomerular and tubular structures may be present in non-albuminuric subjects with T1D (27). These observations have raised significant concern in the use of albuminuria alone as a reliable marker for risk of development of DN. Thus, a major pitfall has been the inability to identify high-risk patients at an early stage for microvascular and other complications. Interestingly, studies have shown that there is no significant difference in serum KL in patients with diabetes without nephropathy compared to healthy controls, whereas

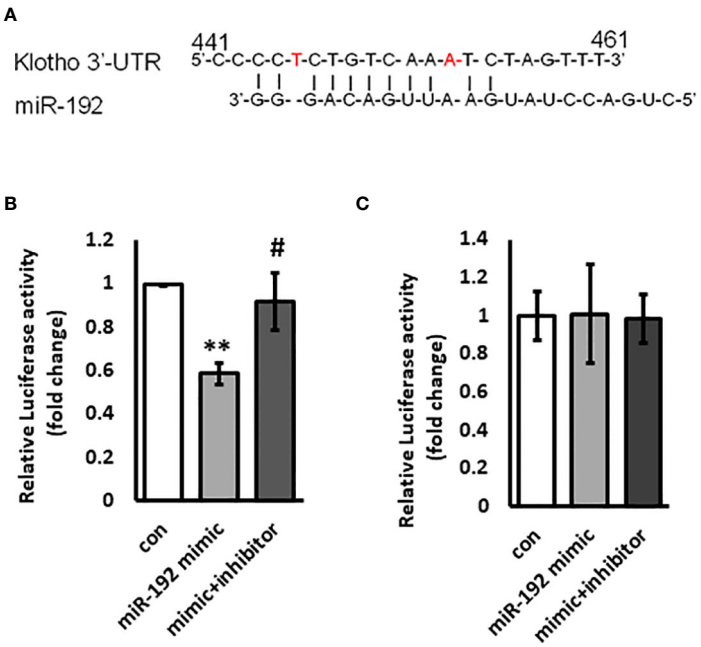


FIGURE 5
KL is a target gene of miR-192. Potential miR-192 binding site on the 3'UTR of KL promoter is shown in **(A)**. miR-192 mimic or scrambled miRNA or the combination of miR-192 mimic and miR-192 inhibitor were co-transfected with kl-wt-luc 3'UTR reporter **(B)** or kl-mut-luc 3'UTR reporter **(C)** into HK2 cells for 72 hours and reporter activity was measured using Dual-Luciferase Reporter Assay kit. **p<0.01 and #p<0.05.

soluble KL is decreased in early chronic kidney disease in diabetic patients (28). Our study shows that KL is negatively correlated significantly with HbA1c, and this is consistent with a previous study from Poland in a pediatric population. Contrary to that same study, we found a significant negative correlation with diabetes duration, likely because our population had a longer duration of T1D (14). Poor glycemic control and prolonged duration of diabetes are the most important risk factors for development of complications, and a decrease in KL levels may reflect early changes at the renal level which may not be identified by albuminuria. This is supported by a recent meta-analysis showing that circulating KL levels were lower even in the very early stages of DN (29). Based on these, circulating levels of KL could be used as an early marker for DN in youth with T1D. Furthermore, overexpression of the KL gene or administration of KL offers beneficial effects in rodent models of various renal diseases (30), including db/db mice (31), suggesting that induction of KL could be a novel therapeutic strategy for treating DN.

Many recent studies have shown that miRNAs play critical roles in DN. Thus, in addition to the circulating levels of KL, we examined the serum levels of multiple miRNAs that had previously been implicated in DN, including miR-192, miR-126, miR-21, miR-29a and miR-210. Of these, only miR-192 is negatively correlated with circulating levels of KL in children with prolonged duration of diabetes, which we expect is associated with an increased risk for diabetic complications. This is broadly consistent with prior observations that levels of miR-192 are negatively correlated with albuminuria in patients with type 2 diabetes (32). Elevations of TGF-β and miR-192 have been shown to lead to increased Col1a2 expression (15, 17) and attenuation of autophagy in glomerular mesangial cells (MMC) *in vitro* (16). It has been shown that TGF-β induces renal fibrosis by increasing miR-192 expression, and miR-192 inhibition blocks TGF-β induced renal fibrosis in a mouse model of chronic kidney disease (33). On the other hand, reduced levels of KL aggravates renal fibrosis in chronic kidney disease (34), and overexpression of KL reduces senescence and oxidative stress, and decreases fibrosis and kidney injury in mice in a model of immune complex glomerulonephritis (11). Our results show a role for miR-192 in oxidative stress, inflammation and senescence, key pathogenic mechanisms in the development of DN. Interestingly, even though both miR-192 and KL have been independently implicated in the pathogenesis of DN, the relationship between miR-192 and KL has not yet been reported. In this study, we show that overexpression of miR-192 inhibits the levels of KL in HK2 cells, and that miR-192 targets KL through its 3'UTR suggesting that KL is a target gene of miR-192.

In summary, our studies provide evidence that in children with T1D, changes in miR-192 and KL correlate with risk factors for complications such as DN, and that changes in KL and/or miR-192 levels could serve as early biomarkers for diabetes complications in youth with T1D. Our *in vitro* experiments provide proof of concept for a role of miR-192 in influencing KL levels, as well as pathophysiological processes such as oxidative stress, inflammation or senescence that play a role in diabetes complications. Limitations of our study include a lack of normal control subjects, and a smaller sample size of subjects with prolonged duration of diabetes. Future longitudinal follow up studies are needed to establish that lower KL levels preceded the onset of DN in our cohort of children with T1D.

## Data availability statement

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

## Ethics statement

The studies involving human participants were reviewed and approved by the Institutional review board at University of Pittsburgh (Protocol Number: PRO15100286). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

ZG, IL, JH, RM-study design. ZG, YL, PP- performed the experiments. ZG, IL, PP, VA, HF, EF, JH, RM- analysis of data and interpretation. ZG, IL, EF, JH and RM- writing the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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

## Publisher's note



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# Precocious puberty and microbiota: The role of the sex hormone–gut microbiome axis

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Puberty is a critical phase of life associated with physiological changes related to sexual maturation, and represents a complex process regulated by multiple endocrine and genetic controls. Puberty is driven by hormones, and it can impact the gut microbiome (GM). GM differences between sex emerge at puberty onset, confirming a relationship between microbiota and sex hormones. In this narrative review, we present an overview of precocious pubertal development and the changes in the GM in precocious puberty (PP) in order to consider the role of the sex hormone–gut microbiome axis from the perspective of pediatric endocrinology. Bidirectional interactions between the GM and sex hormones have been proposed in different studies. Although the evidence on the interaction between microbiota and sex hormones remains limited in pediatric patients, the evidence that GM alterations may occur in girls with central precocious puberty (CPP) represents an interesting finding for the prediction and prevention of PP. Deepening the understanding of the connection between the sex hormones and the role of microbiota changes can lead to the implementation of microbiota-targeted therapies in pubertal disorders by offering a pediatric endocrinology perspective.

KEYWORDS

**precocious puberty (PP), gut, microbioma, sex hormone, axis**

## Introduction

Puberty is a critical phase of life associated with physiological changes related to sexual maturation and represents a complex process regulated by multiple endocrine and genetic controls (1). Gonadal sex hormones are secreted in accordance with the pulsatile secretion of the pituitary gonadotropin follicle-stimulating hormone and luteinizing

hormone (FSH and LH), which is activated by the release of the hypothalamic gonadotropin releasing hormone (GnRH) (2). Rising levels of sex steroid hormones trigger changes in physical appearance during pubertal development.

Pubertal development can be at the appropriate time, precocious or delayed. Abnormal pubertal development can cause considerable distress to the patient and could also be a sign of an underlying pathology.

Puberty is driven by hormones, and it can impacts the gut microbiome (GM) (3). Gut microbial communities represent one source of human genetic and metabolic diversity; they influence nutrient acquisition, brain development, immunity, endocrinology and the nervous system (4–6). Few studies have described changes in the gut microbiome as a function of age. It has been observed that the development of the microbiome from infancy to childhood is dependent on multiple factors, and an association between sex hormones and microbiota has been proposed. GM differences between sex emerge at puberty onset, confirming a relationship between microbiota and sex hormones (3, 7). Some theories suggest that the GM regulates the levels of sex hormones via interactions among its metabolites, the immune system, chronic inflammation and some nerve–endocrine axes, such as the gut–brain axis. Additionally, bidirectional interactions between the microbiome and the hormonal system have also been proposed, and the mechanisms of these interactions are being explored; data are limited in pediatrics (8).

In this narrative review, we presented an overview on the precocious pubertal development and the changes in the GM in precocious puberty in order to consider the role of the sex hormone–gut microbiome axis from the perspective of pediatric endocrinology.

# Methods

A narrative review was presented (9); a non-systematic summation and analysis of the available literature on the topic of the changes in the GM in precocious puberty and the role of the sex hormone–gut microbiome axis was considered. Authors reviewed the relevant English literature on a specific topic, in the past 15 years, including original papers, metanalysis, clinical trials and reviews. Case reports and case series were excluded. A search in PubMed, Scopus, EMBASE, and Web of Science was adopted. The following search terms (alone and/or in combination) were adopted: precocious puberty, timing of puberty, gut microbiome, sex hormones, sex hormone–gut microbiome axis. The authors revised the abstracts of the available literature (n=83) and reviewed the full texts of potentially relevant articles (n=50) that were analyzed and critically discussed. The reference list of all articles was checked to consider relevant studies. The contributions were independently collected by V.R., G.M., C.R. and C.H., critically analyzed by V.C., S.P. and V.C., S.P., C.B., G.Z discussed the resulting draft before finalizing. The final version was approved by all the coauthors.

# Pubertal development

## Physiology of the puberty

Puberty is a crucial developmental milestone characterized by the maturation of gametogenesis (precursor cells undergo cell division and differentiation to form mature haploid gametes), the production of gonadal hormones and the development of secondary sexual characteristics and reproductive functions.

Normal puberty results from prolonged, mature activity of the hypothalamic–pituitary–gonadal (HPG) axis (2). The hypothalamus releases the GnRH in a pulsatile way into the pituitary portal venous system, where it stimulates LH and FSH secretion (pulsatile as well). LH primarily stimulates Leydig cells in the testes and theca cells in the ovary to secrete androgens. FSH primarily stimulates the ovarian follicle or seminiferous tubules to form estrogen, inhibin, and eggs or sperm. The interstitial, tubular and follicular compartments act together through paracrine processes to produce estrogen, and regulate sex steroid production and gamete development. Steroid hormones have endocrine negative feedback effects on GnRH and gonadotropin secretion. FSH secretion is suppressed by the negative feedback of inhibin, progesterone, and estradiol. In adult female subjects, critical estradiol concentration stimulates the LH surge that initiates ovulation.

At birth, due to the absence of placental steroids that suppress the HPG axis, there is an activation of such axis, which causes an increased production of steroidal hormones, defining the first step that will be continued in adolescence. This transient activation starts approximately one week after birth, and it stops after few months (approximately 6 months of age) (10–12).

The HPG axis is not completely dormant throughout childhood, especially in females, who show moderately higher FSH concentrations than males. Sometimes, it is also possible to see ovarian follicles using ultrasounds. During adolescence, the HPG axis undergoes complete reactivation.

The most important GnRH-inhibitory systems are gabaergic (neurons that produce gamma-aminobutyric *acid)* and opioidergic; Kisspeptin, neurokinin B and dynorphin A, three neuropeptides present in the arcuate nucleus (ARC), are considered to be fundamental generators that influence GnRH release, as they contribute significantly to the physiology of puberty in boys and girls (2).

The role of leptin in the physiology of puberty is well known. Leptin is a cytokine produced mainly by adipocytes, which acts as an anorectic factor, playing an essential role in controlling body weight, food intake, and energy balance by inhibiting the

hypothalamic neuropeptide Y (NPY), thus suppressing appetite (13, 14). Normal body weight and composition must be attained during childhood to avoid pubertal dysfunction (1). In addition to the leptin-NPY interaction, some studies showed that leptin acts on puberty and reproductive function by directly interacting with the KiSS-1gene. GnRH neurons lack leptin receptors, but KiSS-1 neurons express them. Leptin directly stimulates kisspeptin release and mediates the pulsatile release of GnRH (15).

Physical changes occurring in puberty in male and female adolescents and the assessment of secondary sexual characteristics (breast buds in girls, testicular volume in boys, pubic hair in both), were classified according to the Marshall and Tanner classification

## Precocious puberty

Puberty is a complex process with wide physiological variation. Mechanisms regulating the onset of puberty involve genetic, nutritional and environmental interactions (1).

Abnormal fetal nutrition along with the endocrine system could lead to developmental alterations that permanently affect structure, physiology and metabolism. Interactions between hormones and nutrition during crucial periods of growth are essential concerning metabolic adaptation response control and pubertal development expectation (16).

An increasing amount of evidence suggests that the prenatal and early postnatal periods represent an important period for the programming of puberty onset (17). Various studies have shown that prenatal exposure to unfavorable environmental factors, such as factors responsible for children born SGA (small for gestational age) and/or IUGR (intrauterine growth restriction), have an effect on puberty timing (18, 19). A child born SGA may undergo several puberty alterations, such as precocious puberty (20–22).

Precocious puberty (PP) is defined by the early appearance of secondary sexual characteristics before the age of 8 years in female adolescents and 9 years in males (23). According to the underlying physiopathological process, pathological PP is classified as follows:

- Central precocious puberty (CPP) or gonadotropin-dependent PP (or true precocious puberty) caused by an early maturation of the HPG axis due to congenital or acquired central nervous system (CNS) lesions or monogenic defects, or it can be idiopathic (1);
- Peripheral precocious puberty (PPP) or gonadotropin-independent PP (or pseudoprecocious puberty), due to an excessive secretion of gonadal sex hormones or adrenal hormones from a genetic or tumoral etiology, germ cell tumors secreting hCG (human chorionic gonadotropin—exclusively in boys), or an exogenous source (1).

It has been evaluated that CPP affects 1 in 5000-10000 children, and is 10 times more common in females than in males (24). In addition, most of the cases of CPP in females seem to be idiopathic (25), whereas a higher prevalence of CPP in males seems to be commonly caused by pathological brain lesions (26). In particular, hypothalamic hamartoma is the most common brain lesion causing CPP.

There are some reports of familial forms, but the genetic basis is not completely understood. Some studies have shown associations with mutations (loss or gain of function) of KISS1 and makorin ring finger (MRF3) genes and their receptors. Mutations in these genes result in CPP; on the contrary, in familial CPP, *MKRN3* defects were found in approximately 30% of families in subjects with apparently sporadic CPP, and *MKRN3* defects were noted in approximately 8% of cases (27).

Patients with PP show accelerated sexual and physical growth concurrently with a growth spurt. If untreated, the accelerated epiphysial growth could lead to a short stature in adulthood due to premature epiphysial closure.

An accurate personal and familiar history, a complete physical examination, hormonal, and radiological exams is crucial in the PP diagnosis (23, 24, 28, 29).

The clinical examination should be focused on the auxological data, the assessment of pubertal signs according to the Marshall and Tanner classification (12, 30), the growth pattern during the last 6–12 months, the rate of progression of pubertal signs and additional signs of puberty (acne, oily skin, erections, nocturnal emissions in boys and vaginal discharge and menstrual bleedings in girls).

The baseline LH level is a promising biomarker to diagnose CPP (31); a basal morning LH value of more than 0.2 mUI/ml is usually considered indicative of puberty (28, 31–37). In addiction, an LH to FSH ratio higher than 0.6 has been associated with CPP (31, 34). Moreover, the GnRH stimulation test remains the gold standard to identify CPP, and the cutoff peak LH level of >5 IU/L is widely used to diagnose CPP (come sopra).

Other hormonal evaluations should include thyroid tests, testosterone, estradiol, 17-hydroxyprogesterone (17-OHP), carcinoembryonic antigen (CEA), Cancer antigen 125 (CA125), alpha-fetoprotein and beta-hCG depending on the patient's history (38).

To define the biological age of the child, a bone age X-ray of the nondominant (left) hand and wrist is taken. An advanced bone age of more than 2.5 standard deviations (SD) or more than 2 years is more likely associated with pathological PP (28, 35).

In girls, pelvic ultrasound is a useful tool to assess the premature pubertal development of ovaries and exclude the presence of ovarian cysts or tumors (39, 40).

Brain MRI is suggested in patients with a CPP diagnosis to rule out CNS lesions (28), that should be performed routinely in young boys (< 6 years) (28).

The most important goals of the PP treatment are to preserve the adult height and to reduce the associated psychosocial stress.

GnRH agonists, with 1-month or 3-month depot formulations, are the standard of care in CPP. GnRH agonist therapy is widely considered safe. The most common adverse reactions include local skin effects and postmenopausal symptoms (35). The periodic verification of pubertal progression, growth velocity, and skeletal maturation is required.

The treatment of PPP varies according to the pathogenesis, and the primary aim of treatment is to eliminate the endogenous or exogenous sources of sex steroids (41, 42). Surgery is indicated in adrenal and gonadal tumors.

## Gut–sex hormones axis

The human intestinal tract is colonized by a large number of microorganisms (circa $10^{13}$-$10^{14}$), known together as "microbiota". The gut microbiome is the human body's major ecosystem, so much so that according to some authors, it represents a "separate organ" (43). The human colon can be populated by more than 1000 different bacterial species and, each host can boast at least 160, which, however, vary in type and quantity depending on the person's own health (44–46). This element underscores the interdependence that exists between host and GM, which affects multiple aspects of host health, particularly endocrine, gastroenterological (digestive function and intestinal permeability), and immune (resistance to foreign pathogens and stimulation of immunity) (47). Microbiota interact with a variety of metabolic and endocrine pathways of the host through genetic expression of more than 100 times the human genome. GM's variety, composition and impact on health depend on a great number of variables, both internal, such as age, genetic factors, gender, and endocrine and immune systems, as well as external factors, such as diet, environment, drugs, and pathogens. All together they influence the delicate balance of the intestinal microecological system. In addition, research has shown that an imbalance in the GM can lead to a range of related diseases, especially those of autoimmune origin (5, 47).

In healthy subjects, more than 90% of bacteria are part of *Firmicutes* or *Bacteroides*' phyla, and alterations in microbiota diversity are related to adverse outcomes in the host's health. A decreased *Firmicutes/Bacteroides* ratio correlates with health issues such as obesity and immunological diseases (4, 48). Some authors have reported an association between an incremented *Firmicutes/Bacteroides* ratio and type 1 diabetes mediated by cell junction disruption with incremental gut permeability, bacterial translocation and the subsequent expression of pro-inflammatory cytokines (49).

The relationship between sex hormones and GM has been widely explored in recent studies and is an expanding research field that may lead to new therapeutic options for a great variety of sex-related diseases; the cluster of the gut microbiome's genes capable of influencing sex hormone levels has been named the "microgenderome".

One of the earliest studies was performed at the cellular level in the 1980s, in which it was found that progesterone promoted the growth of *Bacteroides* species and *Prevotella intermedia* (50, 51). Recently, Yurkovetskiy et al. (52) sequenced bacterial DNA extracted from the cecal contents of prepubertal (4 weeks old) and postpubertal (10-13 weeks old) mice and found that α-diversity was not significantly different between the sexes in prepubertal mice, which was evident among postpubertal mice. Furthermore, by sequencing the 16S rRNA genes of the microbiota of males, females, and castrated males, they observed that the microbiome of females was closer to that of castrated males than that of uncastrated males (50, 52). Among the most recent studies evaluating the relationship between microbiota and sex hormones, Elin Org et al. (53) further demonstrated the effect of androgens on microbiota composition, particularly by assessing the effects arising from gonadectomy and hormone supplementation (53). In contrast, there are still few studies conducted in humans evaluating the relationship between estrogen fluctuation and gut microbiome composition (4, 48). Moreover, these studies have an important limitation dictated by interfering factors, such as genetics and the environment, so most results can only support the existence of a correlation between sex hormones and the microbiome, rather than a causal relationship (50). Koren et al. (54), when sequencing stool samples from 91 women, observed that the gut microbiome was markedly altered during pregnancy, especially during the third trimester, when estrogen peaks, regardless of health status (50, 54). A European study by Mueller et al. (55) showed that healthy males had a higher abundance of *Bacteroides-Prevotella* than fertile females, while the microbiota of postmenopausal women did not differ from that of males (50, 56). Both studies demonstrate how estrogen and related female hormones are crucial in regulating the composition of the gut microbiome.

Therefore, it is well known that microbiota can affect estrogen levels and that, in turn, estrogen levels may be influenced by microbiota composition and diversity. Microbiome is capable of metabolizing estrogens *via* the expression of B-glucuronidase, an enzyme that mediates the deconjugation of dietary and non-dietary estrogen. Unconjugated estrogen can enter the systemic flow and become metabolically active by acting on alpha and beta estrogen receptors, which are expressed in a variety of organs and tissues; estrogen activity has an impact not only on reproductive health but also on cardiovascular risk, metabolic

and bone homeostasis and the central nervous system (49, 50). Microbiota diversity is associated with higher urinary estrogen levels in postmenopausal women and in men, whereas in premenopausal women, estrogen levels do not seem to be influenced by microbiota composition, suggesting that microbiota mostly influences the levels of non-ovarian estrogens. The supplementation of phytoestrogen is capable of promoting gut colonization from specific bacterial species, and a phytoestrogen-rich diet may be associated with a lower risk of metabolic syndrome in Asian postmenopausal women (57, 58).

As already stated, the microbiome also influences the level of androgens in the host's organism, and this might occur through a similar mechanism to that observed in women; a recent study found the levels of non-glucuronidated dihydrotestosterone to be lower in the distal intestine of germ-free mice compared to mice with normal microbiota, thus suggesting that intestinal bacteria express genes capable of metabolizing human sex hormones (59).

In turn, the sex hormone level may also affect microbiome composition: androgen excess, as in PCOS patients, may also lead to dysbiosis and lower bacterial diversity. PCOS is an endocrine disease characterized by higher androgen and lower estrogen levels, and several studies associated intestinal dysbiosis in PCOS patients with lower bacterial diversity, resulting in reduced butyrate production, higher BMIs and higher testosterone serum concentrations. Additionally, the gut microbiome plays an important role in determining insulin secretion by producing SCFAs, which help to reduce the inflammatory response; dysbiosis may lead to insulin resistance and alterations in glucose metabolism, as in polycystic ovary syndrome; higher insulin levels stimulate the ovary in producing androgens, thus perpetuating the pathogenetic mechanism of PCOS (48).

Microbiota and their metabolites may also affect every stage of female fertility, pregnancy, embryo development and timing of delivery, by colonizing the vaginal tract and, according to some authors, the endometrium and placenta. Microbiota's alterations have been associated with the secretion of proinflammatory cytokines and preterm delivery (60). Neonates born from cesarean section delivery show lower diversity in intestinal bacteria, probably because they have not been colonized by maternal intestinal flora by passage through the vaginal tract (48).

Another possible mechanism for explaining microbiota's influence on sex hormone levels involves the "gut-brain axis", the two-way communication pathway between gut and CNS, according to which gut bacteria are an important mediator between the brain and the endocrine system (4). GM is central in modulating the brain–gut axis, and the gut barrier SCFAs, such as acetate, propionate, and butyrate, besides being modulators of inflammation capable of regulating gut motility and wound healing, represent a link between the microbiome and the gut–brain axis (4, 5, 61–63). In addition, the incidence of functional gastrointestinal disorders, such as functional dyspepsia and irritable bowel syndrome, resulting in impaired motility and/or altered sensitivity, are significantly higher in females, presumably due to a complex interaction between sex hormone signaling and stress reactivity in brain–gut axis function (6, 64). Specifically, estrogens have been observed to interfere with gastrointestinal motility and sensitivity through the direct activation of their receptors, located throughout the brain–gut axis, and indirectly through the modulation of other receptor systems (6). Gastrointestinal motility is reduced in women during the follicular phase of the ovarian cycle, when estrogen levels are high (6, 65). Furthermore, supporting the hypothesis that circulating female hormones play an important role in delayed gastric emptying, hormone replacement therapy administered to pre- and postmenopausal women correlates with a slower rate of gastric emptying than that of postmenopausal women not receiving hormone therapy, which in turn is similar to that of men of the same age (66). In contrast, testosterone, or androgens in general, appear to have no effect on gastric motility or gastric hypersensitivity (67, 68).

Estrogens implement their long-term mechanism of action through actions on nuclear receptors and rapid, nongenomic action through the activation of estrogen receptor 1 (ER 1) receptors coupled to membrane G proteins (6, 6). ERs are ubiquitously expressed in the CNS and in pathways involved in visceral pain perception, including the hypothalamus, amygdala, and midbrain, all of which have been shown to send extensive projections to vagal neurons involved in the modulation of gastrointestinal function (6, 69–71). Specifically, in peripheral visceral afferents, estrogen appears to modulate nociception by altering the opening of ion channels and the regulation of receptor expression, as well as activating the cholic tachykinin neurokinin 1 receptor and inducing the release of substance P (6, 72, 73). In addition to interfering with pain modulation, finally, estrogen is involved in visceral information processing in the CNS. Cerebral imaging studies have found that, in comparison with males with IBS, females with IBS display a greater activation of emotional circuits, including the amygdala and locus coeruleus, in response to adverse visceral stimuli (74).

Furthermore, several studies have demonstrated better cognitive functions and reductions in psychiatric symptoms in selected patients treated with fecal transplant. More studies on humans are needed to better understand the underlying mechanisms of this axis (4, 75).

In Figure 1, the sex hormone–gut microbiome axis is shown.

## Microbiome in physiological pubertal development

It is well known how a gradual change in microbiota composition occurs with age with a general reduction in the

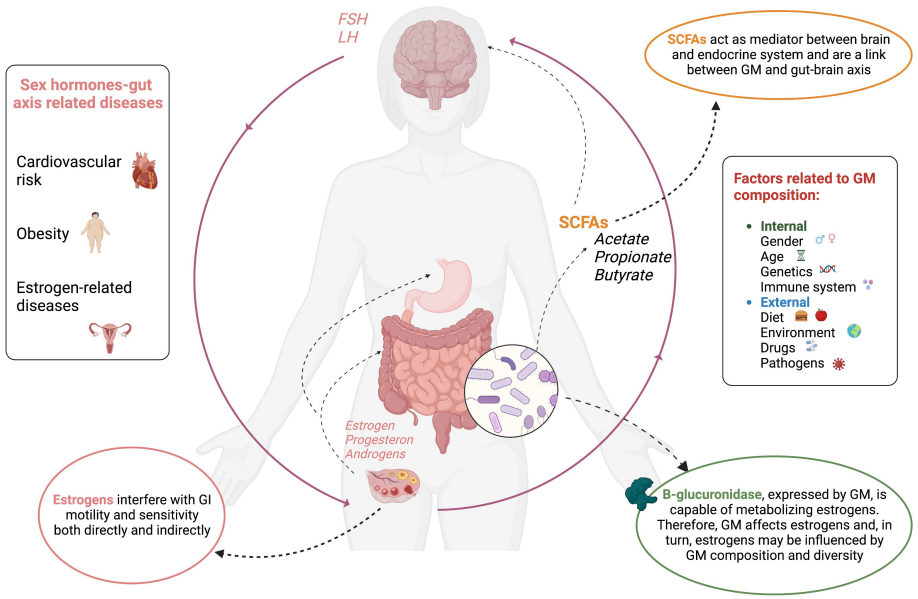


FIGURE 1
Interconnections between sex hormone and gut microbiome.

number of aerobes and facultative anaerobes and an increase in the populations of obligate anaerobic species. Traditionally, the common idea is that between one and two years of age, the human GM starts to resemble that of an adult, which is dominated by species from phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria*. However, many differences were observed at the genus level between adolescent and adult fecal microbiota (3, 7).

Agans et al. (7) conducted a study to assess the distal GM of adolescent children, which showed that abundance of members of *Bifidobacterium* and *Clostridium* genera, species known to colonize the newborn gut and to decrease gradually between 2 and 18 years of life, was statistically significantly higher in adolescent children than adults. The prevalence of these genera had not been recognized previously among preadolescent and adolescent age groups (7).

In 2020, Yuan et al. (76)conducted a cross-sectional study analyzing the biodiversity of the GM at different puberty stages (5-15 years) through 16S rRNA sequencing. No difference in alpha or beta diversity between non-pubertal and pubertal subjects was found, but the study evidenced differential bacterial taxa between the two groups. In particular, non-pubertal subjects were characterized by mainly micro organisms belonging to the order *Clostridiales*, family *Costridiaceae*, and genus *Coprobacillus*. On the other hand, the puberal group showed a higher prevalence of class *Betaproteobacteria*, order *Burkhollderiales* (76). Further analyses of the association between serum sex hormones and bacterial abundance were conducted. The results highlight that the level of testosterone was associated with the abundance of *Adlercreutzia, Dorea, Clostridium* and *Parabacteroides* genera. Authors hypothesized that these bacteria might be affected by sex hormones (76). Several studies have investigated the connection between gut microbes and sex steroid hormones. Shin et al. (77) demonstrated a relationship between intestinal bacterial community profiles and testosterone/estrogen status in humans: *Acinetobacter, Dorea, Ruminococcus* and *Megamonas* were significantly correlated with testosterone levels, while *Slackia* and *Butyricimonas* correlated significantly with estradiol levels (77).

It has been reported that GM does not seem to be affected by gender in children, but differences emerge at puberty onset (59). Microbiota-related diseases also show a gender bias, supporting that the relationship between intestinal bacteria and gender may be biunivocal (4). Although data on adolescents' GM are still limited, a recent cross-sectional survey found that the distinction of the GM between the two sexes becomes more marked at puberty (78, 79). Comparing teens' and adults' microbiota, it was found that the amounts of bifidobacteria, in particular, decreased with age in several studies (7, 80–82), and age-related associations with *Bacteroidetes* and *Firmicutes* (80–82)were also reported (79). Hollister et al. (81) compared puberal and adult GM composition, pointing out that, during pubertal development, GM in girls changes progressively, increasingly resembling that of adult women, directly proportional to their pubertal developmental degree. In both males and females, dominant taxa are *Clostridia* and *Bacteroidia*, and the major element suggesting maturation of microbiota is represented by a

change in the dominance of the *Clostridiales* and *Bacteroidales* classes of bacteria. Indeed, during puberty, the relative abundance of *Clostridiales* gradually increases and that of *Bacteroidales* decreases, gradually resembling the composition of the adult microbiota. In terms of phylum, the abundance of *Firmicutes* increased as puberty progressed, while the abundance of *Bacteroidetes* decreased. However, although Hollister et al. (81) observed GM changes in both boys and girls, only in female adolescents statistically significant data were obtained, reasonably due to later males' pubertal development (81).

These data were confirmed by Yuan et al. (78) who determined the characteristics of the GM of both genders at different pubertal status. The GM was analyzed in 89 Chinese participants aged 5–15 years. Participants were divided into prepuberty and puberty groups for both males (n=49) and females (n=40). This cross-sectional study revealed that sex differences in the GM composition and predicted metabolic profiles existed before puberty and it became more significant in puberty. Specifically, results indicate that *Dorea, Megamonas, Bilophila, Parabacteroides* and *Phascolarctobacterium* genera represent microbial markers for pubertal subjects (78). They suggested that sex-dependent GM diversity is, in part, due to sex hormones, and, in part, to other non-hormonal influencing factors (78).

## Gut microbioma and precocious puberty

Considering evidences on the role of the GM during physiological pubertal development, their role in pathological puberty is becoming of increasing interest. As reported (83), timing of puberty can be influenced by GM, particularly by certain *Clostridia* species, including species of the genera *Ruminococcaceae Faecalibacterium* and *Ruminococcus*, which regulate host sex hormone levels. Specifically, these species affect estrogen metabolism through their beta-glucuronidase activity (81). The beta-glucuronidase enzymes of *Ruminococcus* and *Faecalibacterium* spp. are able to cleave both estrone and estradiol, whereas *Bacteroides* species are only capable of metabolizing estrone. Therefore, estrone-estrogen metabolite ratio in urine correlates positively with the relative abundance of *Ruminococcus* and negatively with that of *Bacteroides* spp (81, 84). According to these data, it is conceivable that the GM may partly regulate the onset of puberty through its estrogen metabolism. However, as much as the GM, through specific gut microbes capable of metabolizing estrogen, seems capable of regulating puberty, the reverse may also be possible. In fact, sex hormones could directly affect the growth of specific taxa by directing the maturation of the gut microbiota (81).

Furthermore, recent studies have observed that metabolites produced by the GM could influence the human endocrine system, activating the enteric nervous system. Some of the best studies on microbiota functions highlight how gut microorganisms provide energy to the host through the production of short-chain fatty acids (SCFAs), including butyrate (the most abundant SCFA) and propionate, both of which participate in bile salts metabolism and play an important role in brain-gut axis (4, 48). It has been demonstrated that Free Fatty Acid Receptor (FFAR) 2 and FFAR3, endogenous receptors, interact with the SCFAs and have been shown to be expressed in enteroendocrine cells that produce peptide YY, an anorectic hormone, with consequent involvement in the regulation of the host energy, appetite, adipose tissue stores and hormonal balance, influencing puberty timing (85, 86)

Indeed a close association between obesity and puberty has been found; in particular, PP has been positively related with body mass index (BMI). On the basis of this information and given that children affected by PP tend to be obese, it has been hypothesized that GM could be involved in the pathogenesis of PP. The study conducted by Dong et al. (87) elucidated differences in the GM between patients with idiopathic central precocious puberty (ICPP) (n=25) and healthy girls (n=23). Authors applied 16S rDNA sequencing to compare the GM between two groups. They observed that the gut genera identified in ICPP are similar to those that are associated with obesity, in particular, *Rumicoccus Gemmiger, Oscillibacter* and *Clostridium XIVb*. Considering microbial species levels, girls with ICPP were enriched in *Rumicoccus bromii, Ruminococcus gnavus*, and *Ruminococcus leptum.* The first two were found in obese populations; they could promote the energy absorption and hyperplasia of adipose tissue, while *Ruminococcus leptum* was reported to influence human weight changes (87–89). These results highlight the association among obesity, ICPP and GM dysbiosis. The authors hypothesized that GM dysbiosis leads preadolescent girls to a process similar to that in obese patients and that the proliferation and deposition of adipocytes trigger precocious puberty. However, intestinal dysbiosis could also induce the earlier activation of the hypothalamic–pituitary–gonadal axis (HPGA) (87).

The involvement of the GM in the mechanism of secretion of estrogen, FSH and LH has been investigated in different studies, but it is still unclear. A previous study indicated a relationship between estrogen and bacteria, such as *Clostridia* and *Ruminococcaceae*, and a significant microbial differences among the control group and ICPP (90). Dong et al. (87) explored the relationship between three clinical biomarkers (FSH, LH and insulin resistance) and the GM. Considering ICPP girls, authors demonstrated a positive correlation between FSH and *Fusobacterium*, and LH and *Gemmiger*, and a negative correlation between LH and *Romboutsia* (87). In addition, insulin resistance has been positively correlated with *Gemmiger, Ruminococcus, Megamonas* and *Bifidobacterium* (87).

In female puberty onset, the important role of leptin is well known. Leptin is an adipocyte metabolic peptide, and the gene involved in its expression is correlated with SCFAs (83, 85, 88, 91, 92).

The close association among the GM, hormone secretion and obesity inspired the study of the mechanism of the GM in triggering CPP. ICPP girls investigated in the work by Dong et al. were characterized by microbes associated with SCFA production: *Ruminococcus bromii, Ruminococcus callidus, Roseburia inulinivorans, Coprococcus eutactus, Clostridium sporosphaeroides* and *Clostridium lactatifermentans*. The relationship between SCFA production and ICPP is explained through the mechanism induced by a high concentration of SCFAs to the expression of the leptin gene, which activates the HPGA, which, consequently, leads to the onset of puberty (87).

Li et al. (93) enrolled 27 CPP girls, 24 overweight girls and 22 healthy controls to explore the connection between obesity and CPP. This study showed that CPP patients exhibited overrepresented *Alistipes, Klebsiella* and *Sutterella*, which are normally present in patients with neurological diseases. These microorganisms produce metabolites with neurotransmission activity (serotonin and dopamine), which trigger the earlier onset of puberty activating HPGA. The authors identified *Prevotella* both in CPP and in the overweight group; branched-chain amino acid production could promote insulin resistance. This mechanism could explain the high occurrence of obesity in CPP patients (93). In addition, in both groups, elevated nitric oxide synthesis was observed, which is an important gas neurotransmitter that stimulates the secretion of gonadotropin-releasing hormone and promote insulin resistance (93). These conditions, the altered expression of the GM, could explain the link between CPP and obesity (93), as shown in Figure 2.

As reported (1), the macronutrient food content, such as high fat intake, may modulate the premature activation of the HPG axis, inducing precocious activation of puberty. Recently, in experimental model Bo et al. (94) showed that the effect of high-fat diet (HFD) on precocious puberty is regulated by the interaction of gut microbiota and hormones. HFD after weaning caused PP, increased serum estradiol, leptin, deoxycholic acid and GnRH in the hypothalamus (94). In particular, GnRH was positively correlated with *Desulfovibrio, Lachnoclostridium, GCA-900066575, Streptococcus, Anaerotruncus*, and *Bifidobacterium*, suggesting that these bacteria may have a role in promoting sexual development (94). Additionally, the authors (94) reported that "HFD-microbiota" transplantation promoted the PP of mice, supporting that GM modulates local and systemic levels of sex steroids promoting precocious puberty.

## Conclusions

Bidirectional interactions between the GM and the sex hormones have been proposed in different studies. During puberty, the somatic developmental changes are predominantly driven by hormones; therefore, this dynamic and transitional period represents an opportunity to assess the impact of

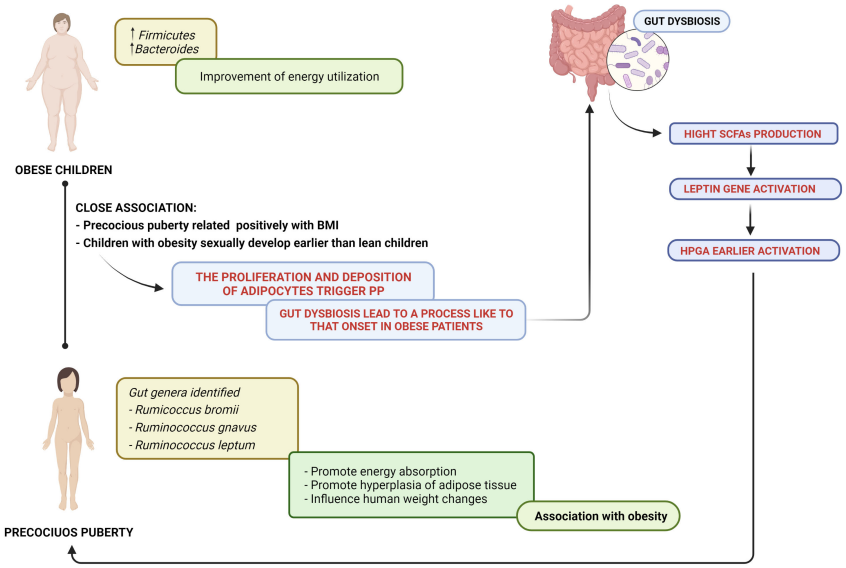


FIGURE 2
Precocious puberty–gut microbiome and obesity: a close association. SCFAs, short-chain fatty acids; HPGA, hypothalamic–pituitary–gonadal axis.

potential hormonal effects on the GM. Although the evidence of the interaction between microbiota and sex hormones remains limited in pediatric patients, the evidence that diversity of the GM at different puberty stages exists and that GM alterations may occur in girls with CPP represents an interesting finding for the prediction and prevention of precocious pubertal development. Deepening the understanding of the connection between the sex hormones and the role of microbiota changes can lead to the implementation of microbiota-targeted therapies in pubertal disorders by offering a pediatric endocrinology perspective.

## Author contributions

VC, VR, GM, CR, CH, SP, CB, GZ participated in the study design, project management, and supervision. VC, VR, GM, CR, CH were responsible for the conceptualization and design of forms, data management, writing, and editing. VC, SP, CB, GZ supervised the manuscript. All authors were contributed to this article and approved the submitted version.

## Conflict of interest

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

## Publisher's note



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# Clinical application of artificial intelligence in longitudinal image analysis of bone age among GHD patients

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**Objective:** This study aims to explore the clinical value of artificial intelligence (AI)-assisted bone age assessment (BAA) among children with growth hormone deficiency (GHD).

**Methods:** A total of 290 bone age (BA) radiographs were collected from 52 children who participated in the study at Sun Yat-sen Memorial Hospital between January 2016 and August 2017. Senior pediatric endocrinologists independently evaluated BA according to the China 05 (CH05) method, and their consistent results were regarded as the gold standard (GS). Meanwhile, two junior pediatric endocrinologists were asked to assessed BA both with and without assistance from the AI-based BA evaluation system. Six months later, around 20% of the images assessed by the junior pediatric endocrinologists were randomly selected to be re-evaluated with the same procedure half a year later. Root mean square error (RMSE), mean absolute error (MAE), accuracy, and Bland-Altman plots were used to compare differences in BA. The intra-class correlation coefficient (ICC) and one-way repeated ANOVA were used to assess inter- and intra-observer variabilities in BAA. A boxplot of BA evaluated by different raters during the course of treatment and a mixed linear model were used to illustrate inter-rater effect over time.

**Results:** A total of 52 children with GHD were included, with mean chronological age and BA by GS of $6.64 \pm 2.49$ and $5.85 \pm 2.30$ years at baseline, respectively. After incorporating AI assistance, the performance of the junior pediatric endocrinologists improved ($P < 0.001$), with MAE and RMSE both decreased by more than 1.65 years (Rater 1: $\Delta MAE = 1.780$, $\Delta RMSE = 1.655$; Rater 2: $\Delta MAE = 1.794$, $\Delta RMSE = 1.719$), and accuracy increasing from approximately 10% to over 91%. The ICC also increased from 0.951 to 0.990. During GHD treatment (at baseline, 6-, 12-, 18-, and 24-months), the difference decreased sharply when AI was applied. Furthermore, a significant inter-rater effect ($P = 0.002$) also vanished upon AI involvement.

Abbreviations

BA, bone age; BAA, bone age assessment; AI, artificial intelligence; GS, gold standard; GHD, growth hormone deficiency; GH, growth hormone; cGHD, complete growth hormone deficiency; pGHD, partial growth hormone deficiency; RMSE, root mean square error; MAE, mean absolute error; ICC, intra-class correlation coefficient; GP, Greulich-Pyle; TW, Tanner-Whitehouse; CNN, convolutional neural network; FLOPS, floating point operations per second; CH05, China 05.

**Conclusion:** AI-assisted interpretation of BA can improve accuracy and decrease variability in results among junior pediatric endocrinologists in longitudinal cohort studies, which shows potential for further clinical application.

KEYWORDS

artificial intelligence, bone age assessment, growth hormone deficiency, children, China 05 bone age standard

# Introduction

Bone development displays certain age characteristics and crucial indicators that could directly reflect the level of maturity and biological age of an individual. Clinically, bone age assessment (BAA) is commonly used to diagnose and monitor growth disorders and endocrine abnormalities in children, including growth hormone deficiency (GHD), hypothyroidism, and precocious puberty, among others. Bone age (BA) can also be used in predicting adult height. It can even be a determining factor in whether a therapy is deemed necessary for patients with central precocious puberty (1, 2). With a whole host of practical applications, BAA has become a common clinical practice among pediatric endocrinologists.

Several methods are used globally in the evaluation of based BA on the left hand-wrist radiograph, including the Greulich-Pyle (GP) atlas and the Tanner-Whitehouse (TW) method (2, 3). Because these methods were largely developed based on a white population, an alternate method geared more toward the Asian population was developed by the Chinese Bone Development Survey Group. The China 05 (CH05) BA evaluation standard, which was formulated from 2003 to 2005 based on children from upper-middle backgrounds in developing cities around China, is now recommended by many experts as more suitable for evaluating Chinese children than the aforementioned methods (4–7).

As useful as it may be, the application of BAA comes with its drawbacks and limitations. Besides being time-consuming and challenging to master as a skill, the results of manual BAA highly depend on the clinician's level of experience. This can result in gaps between the results of BAA performed by junior- and senior-level clinicians, even when using the same set of radiographs. These problems will need to be addressed to ensure the accuracy of BAA, as it affects the diagnosis, monitoring, and treatment strategies of a number of diseases (7, 8).

In recent years, research on BAA has entered a new era with the arrival of artificial intelligence (AI). Several AI systems have already been developed to assess BA in North America and South Korea. These AI systems reportedly yielded both high accuracy and improved time-efficiency compared to manual assessment. However, most of the systems were based on the GP atlas or the TW3 method, which may not be applicable for the Chinese population (9). Although there has been some research involving AI BAA, to date, it has mainly comprised cross-sectional studies (9–11). To the best of our knowledge, no study has been done thus far to explore the performance of AI in longitudinal BA evaluations for endocrine disease, though it's sheer significant and common in clinical scenarios. This study, then, aimed to compare the accuracy and consistency of BAA among pediatric endocrinologists in the absence and presence of AI assistance during the course of treatment in children with GHD.

# Methods

## Participants

A total of 52 patients with GHD were prospectively enrolled between January 2016 and August 2017 at Sun Yat-sen Memorial Hospital. Study participants were treated with PEGylated recombinant human growth hormone for at least one year with a median follow-up period of 24 (IQR: 24–42) months. Inclusion criteria included the following: (1) the child's height at the first visit was below the third percentile of the growth curve for normal healthy children of the same age and sex; (2) the child's annual height velocity $\leq$5.0 cm/year; (3) the peak values of growth hormone (GH) stimulation tests of two different drugs <10.0 ng/ml, including complete GHD (cGHD) with a GH peak <5 ng/ml and partial GHD (pGHD)—GH peak in 5–10 ng/ml, and serum GH was measured using a solid-phase, 2-site chemiluminescent immunometric assay, Immulite 2000, and growth hormone assays were calibrated to NIBSC IS reference standard (98/574); (4) BA was below 9 years for girls or 10 years for boys and was more than one year behind chronological age; (5) prepubertal but $\geq$3 years old. Exclusion criteria included the following: (1) participants who did not follow up as scheduled or did not take the medication as directed (including injection dose and injection frequency); (2) those with any chronic disease or treatment that might interfere with growth or other indicators of efficacy and safety (such as the use of GnRH agonists, protein assimilation drugs, or long-term use of corticosteroids/traditional Chinese medicines, etc.); (3) subjects with poor compliance or who were unable to complete the interview as required by the study. Study participants' demographic characteristics, biochemical indicators, and BA radiographs were measured and monitored every 6

months. To compare the performance of BA readings longitudinally between pediatric endocrinologists and AI, additionally excluded were the following: (1) images with incorrect positioning (i.e., right hand) or with incomplete hand or wrist structures (such as no metacarpal bone, phalanx bone, carpal bone, or 3–4 cm of the distal shaft of the ulna and radius); (2) radiographs from patients who were followed up with for less than one year. Overall, 290 radiographs from 52 patients were collected and assessed. The flowchart of case selection is presented in Figure 1. The study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital (Ethics number: 2015–30), and all patients and their parents provided informed consent before participating.

## Inspection method

All radiographs were acquired using a YSID DR (Siemens, Germany) machine. The left palm of the subject was placed downward and gently pressed against the scanning platform, with all five fingers naturally separated. The angle between the thumb and index finger was about 30°, while the axis of the

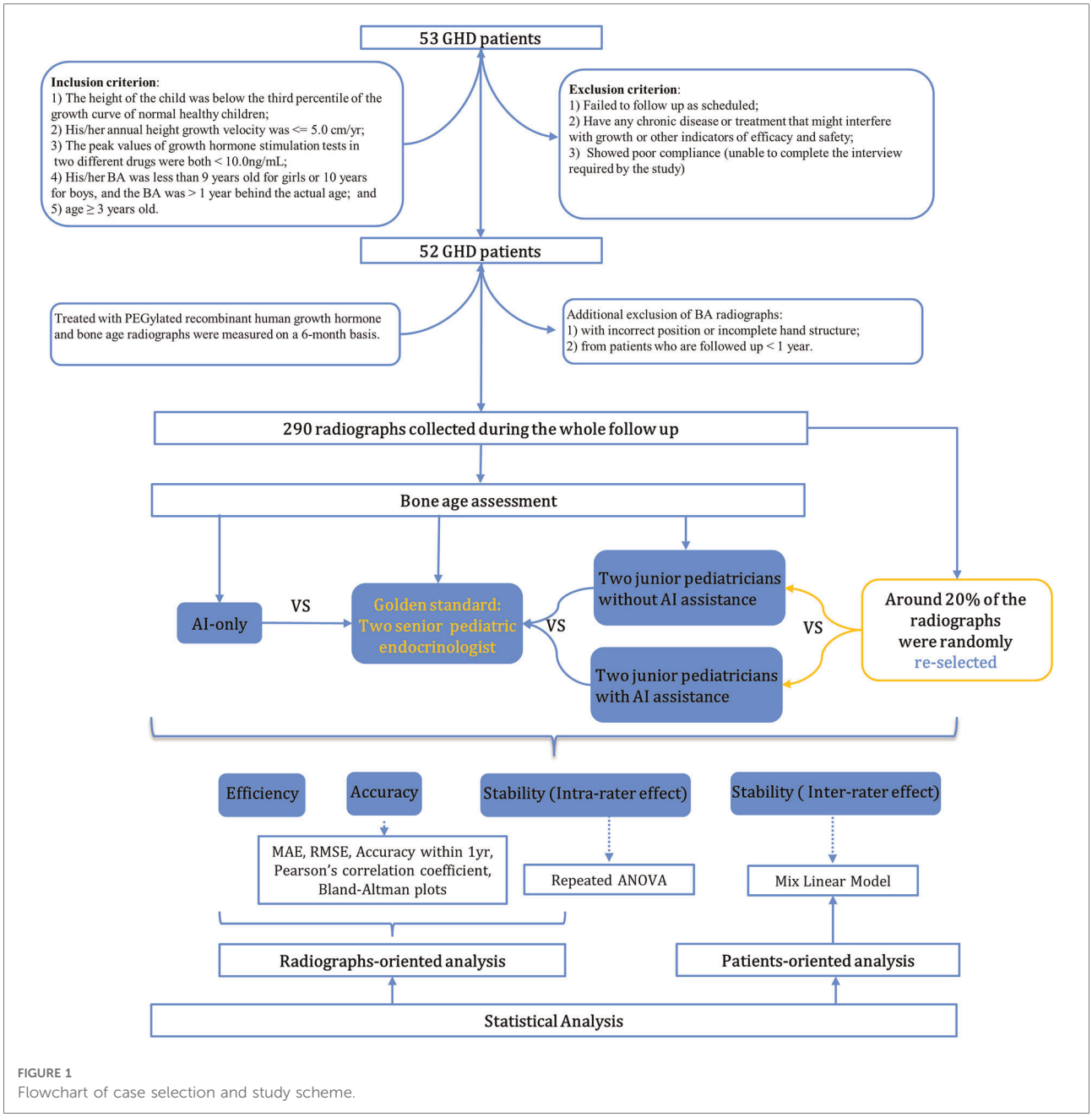


FIGURE 1
Flowchart of case selection and study scheme.

middle finger aligned with the forearm (the arm remained flat and could not be lifted), with a center line that was perpendicular to the head of the third metacarpal and a segment distance of 80 cm. A bandage was deployed if the subject was not able to position his or her hand properly without further assistance.

## AI system of BAA

As illustrated in **Figure 2A**, the AI system (YITU Healthcare Technology Co., Ltd., China) used in this study comprised an alignment module and a subsequent classification module. Both modules were built on a deep residual network (ResNet), a deep convolutional neural network (CNN) with 50 layers and approximately $3.6 \times 10^9$ floating point operations per second (FLOPS). The model was implemented using an open-source machine learning library (TensorFlow version 1.4.1; Google, Mountain View, CA, United States). The left hand and wrist images were automatically processed by the AI system, with targeted bones located, classified, and labeled (such as the radius, ulna, and metacarpals), maturity level evaluated, and BA calculated accordingly. It should be noted that the AI system supports BAA through the TW3 method (comprising 13 bones, i.e., the radius, ulna, and short finger bones), the RUS system (comprising 7 carpal bones), and the CH05 standard (6), since regions of interest may differ according to different medical scenarios. In the present study, AI-aided BAA was performed according to the CH05 standard, as it is more adapted to and thus preferred for assessing the skeletal growth patterns of Asian children (6). When AI-assisted BAA was conducted, the maturity level of each targeted bone was first estimated by AI, and then human raters could modify the outcome at will (**Figure 2C**). Likewise, manual BA evaluation was conducted on the same AI platform. The targeted bones were labeled by AI, but the maturity levels were assessed by raters alone without AI involvement (**Figure 2B**).

## Gold standard of BA

A total of 290 radiographs from the 52 study participants were randomly shuffled and independently evaluated by two senior pediatric endocrinologists (LLY and MZ, each with more than 15 years of experience in pediatric endocrinology) based on the CH05 standard, respectively. They were blinded to patient information, including age, gender, follow-up periods, and previous BA reports. Any inconsistent assessments of BA were re-evaluated, discussed, and confirmed with final consent from both raters. This rating outcome was referred to as the gold standard (GS).

## BAA with and without AI

Similar to the abovementioned procedure, two junior pediatric endocrinologists (ZLN and HLL, each with 5 years of experience in pediatric endocrinology) independently yet concurrently assessed the BA of the 290 radiographs. The slight distinction between their assessments and those of the senior pediatric endocrinologists involved the absence or inclusion of the AI-based BA evaluation system. Six months later, around 20% of the images were randomly selected to be re-evaluated by the junior pediatric endocrinologists both with and without AI assistance to measure intra-rater consistency over time. The rating process was not time-restricted, but the overall length of each evaluation was automatically recorded by the system. Here presents the detailed reading process of BA. Without AI: Based on standard of the CH05, readers evaluate the 13 ossification centers (i.e., radius, ulna, the metacarpals and the proximal, middle and distal phalanges of the first, third and fifth digits) and rate for them. The final bone age is calculated automatically with a weighted coefficient based on CH05. With AI: Before raters' evaluation, AI has rated the 13 ossification centers already, and then raters begin to read and rate on the basis of AI's score according to atlas of CH05. When discrepancy appears, raters would re-compare with the standard atlas of the CH05 and decide to revise or remain the score accordingly. Finally, the bone age is automatically calculated as well. As shown in **Figure 2C**, the reader rated 4 points for the fifth metacarpal bone based on the CH05 standard without AI assistance. However, AI rated it as 4(2). A final score of 4(2) points was determined by the reader after re-checking with the CH05 standard atlas. In this way, AI can help to improve the accuracy of BAA among less experienced clinicians.

## Statistical analysis

Clinical characteristics of the patients were described *via* mean and standard deviation (for normally distributed variables), interquartile range [median (Q25–Q75)] (for variables with non-normal distribution), or frequency and percentages (for categorical variables). The statistical analysis in this research was divided into two parts: (1) radiograph-oriented analysis to explore the accuracy, intra-rater effect, and efficiency of BAA; and (2) patient-oriented analysis to measure inter-rater variation during the course of GHD treatment. Several statistical variants were used to assess the BA divergence between the GS and the manual outcomes from the raters (or AI-only or AI-assisted) in radiograph-oriented analysis, including root mean square error (RMSE), mean absolute error (MAE), and accuracy within 1 year (%). Specifically, accuracy was defined as the percentage of the

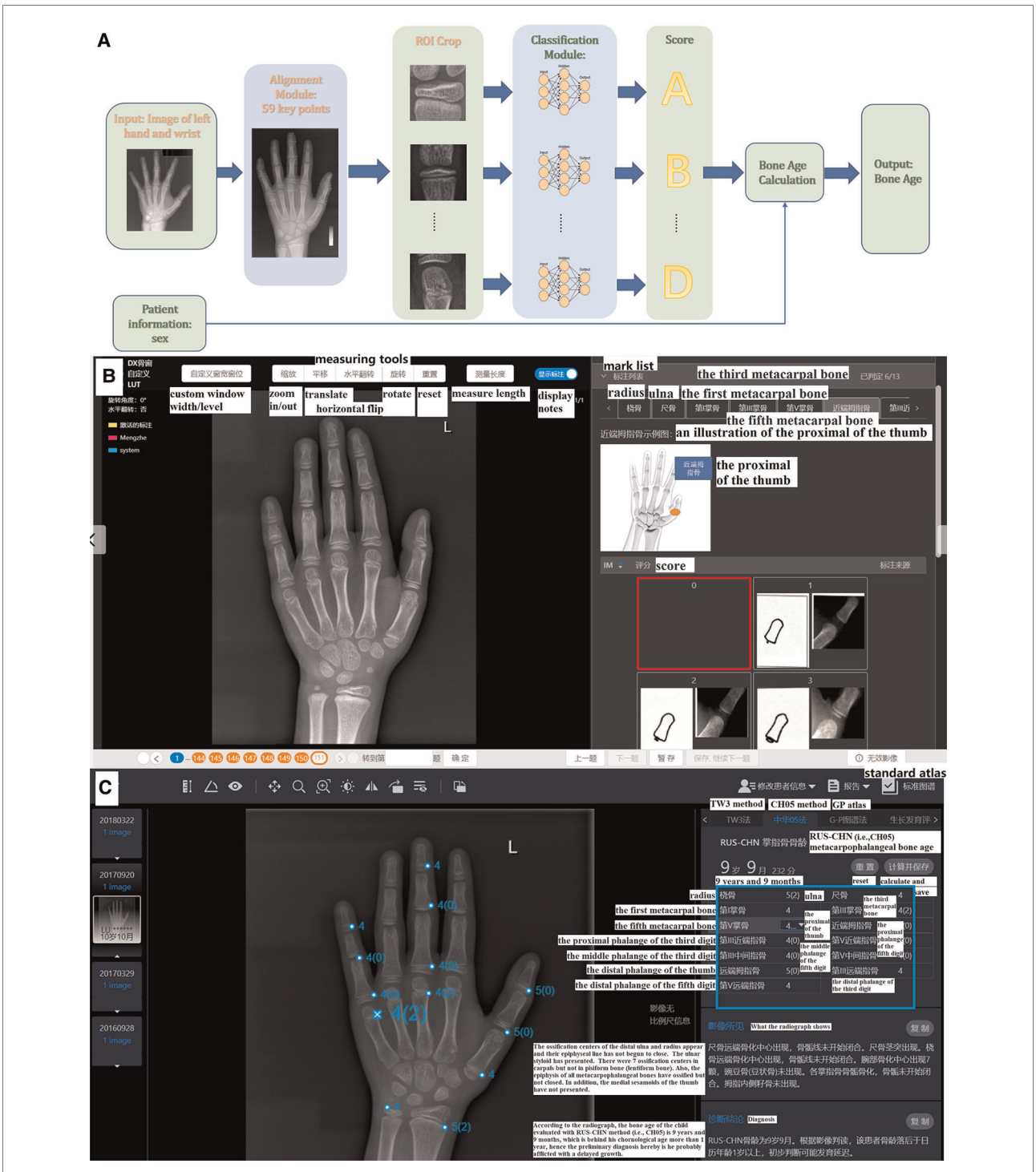


FIGURE 2
(**A**) Network structure of the region-based convolutional neural network. Two modules, including alignment and classification modules, adopted a deep residual network (ResNet) to evaluate bone age automatically. (**B**) The product window for doctors to evaluate bone age without AI assistance. Taking the proximal of the thumb as an example, the rater compares the patient's radiograph shown in the middle of the picture (by using the measuring tools in the upper middle such as zoom in/out) with the standard atlas (in the bottom right) and choses 0–8 points (marked as a red box) for it accordingly. In total, there are 13 ossification centers (i.e., the mark list as shown in the upper right including radius, ulna and so on) need to be scored and the bone age will be calculated automatically. (**C**) The product window for doctors to confirm and modify bone age with AI assistance. AI has scored the 13 ossification centers before raters' evaluation (as presented in the blue box), and then raters begin to check each center according to atlas of CH05 which would show with a click of the button of standard atlas. When discrepancy appears, raters would re-compare with the standard atlas and decide to revise or remain the score accordingly. Finally, bone age is automatically calculated after a click of save button. Here the reader tends to score 4 points for the fifth metacarpal bone based on CH05 while it rated as 4(2) by AI, and a final 4(2) points was decided after a re-check with the standard atlas by the rater.

differences within 1 year. A paired *t*-test was used for MAE/RMSE, whereas McNemar's Chi-square test was used to check whether significant changes in those metrics were observed with or without AI assistance. Additionally, Pearson's correlation coefficient was calculated to measure their relativity, while Bland-Altman plots were generated to demonstrate the mean and 95% confidence interval of the differences. Fisher's *r* to *z* transformation was also performed, and a *Z*-test was used to compare the correlation coefficients. Furthermore, the intra-class correlation coefficient (ICC) based on two-way random ANOVA was used to assess inter-rater variation amongst the pediatricians with and without AI assistance as a measure of variability, while one-way repeated ANOVA was used to quantify the intra-rater effect between both instances of BA evaluation. As for patient-oriented analysis, a boxplot of BA evaluated by different raters during the course of treatment and a mixed linear model were used to illustrate inter-rater effect over time.

A two-tailed *P*-value of less than 0.05 was considered as statistically significant, and Bonferroni correction was applied if a statistical method was used multiple times. All analyses were conducted with R-3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

# Results

## Demographic characteristics of patients

A total of 52 children with GHD were included in this study. The mean chronological age of participants was 6.64 ± 2.49 years, while the mean BA as determined by GS was 5.85 ± 2.30 years at baseline (Table 1). Almost 6 in 10 (59.6%) subjects were girls whose mean height was 104.21 ± 13.39 cm, while the remainder were boys with a mean height of 112.49 ± 13.12 cm. Partial GHD accounted for 75% ($N = 39$) of all GHD cases.

TABLE 1 The demographic characteristics of the 52 GHD patients at the baseline.

| Level | Overall | Boys | Girls | *P* |
|---|---|---|---|---|
| *n* (%) | 52 | 21 (40.4) | 31 (59.6) | |
| Age [mean (SD)] | 6.64 (2.49) | 7.44 (2.54) | 6.10 (2.34) | 0.039 |
| Height [mean (SD)] | 107.55 (13.78) | 112.49 (13.12) | 104.21 (13.39) | 0.036 |
| Weight [mean (SD)] | 17.10 (5.08) | 18.91 (5.38) | 15.87 (4.56) | 0.039 |
| BMI [mean (SD)] | 14.45 (1.13) | 14.62 (1.18) | 14.33 (1.10) | 0.358 |
| Ht SDS [mean (SD)] | −2.67 (0.85) | −2.60 (0.70) | −2.72 (0.96) | 0.918 |
| BA by GS [mean (SD)] | 5.85 (2.30) | 6.57 (2.68) | 5.36 (1.90) | 0.159 |
| GHD type (%) | | | | |
| pGHD | 39 (75.0) | 16 (76.2) | 23 (74.2) | 1.000 |
| cGHD | 13 (25.0) | 5 (23.8) | 8 (25.8) | |

Ht SDS, height standard deviation scores; BA, bone age; GS, gold standard; cGHD, complete growth hormone deficiency; pGHD, partial growth hormone deficiency.

## Accuracy of BAA and AI effect on readers' performance

As presented in Table 2, the MAE and RMSE under AI assistance were 0.489 and 0.757 years, respectively, with an accuracy within one year of 91.03%, while the MAE and RMSE of both independent raters were more than two years off with an accuracy rate of around 10% (Rater 1: 8.36%; Rater 2: 13.45%). In addition, as shown in Figure 3, there was a significantly higher correlation (comparison $P < 0.001$) between AI-derived BA and the reference values ($r = 0.957$) than the BA as assessed by the two raters and GS ($r = 0.668$ and $r = 0.688$).

With the mean BA values approaching to GS more clearly (for example, the BA by Rater 1 decreased from 7.737 ± 3.205 to 7.368 ± 2.562 when the GS is 7.468 ± 2.591), the performance of the two readers improved (all had $P < 0.001$) under the aide of AI, MAE and RMSE both decreased by more than 1.65 years (Rater 1: ΔMAE = 1.780, ΔRMSE = 1.655; Rater 2: ΔMAE = 1.794, ΔRMSE = 1.719) while accuracy

TABLE 2 Accuracy of BA assessment from different raters and effect of AI on raters' performance.

| | BA (mean ± SD, years) | | MAE (years) | | RMSE (years) | | Accuracy within 1 year (%) | |
|---|---|---|---|---|---|---|---|---|
| | Without AI | With AI | Without AI | With AI | Without AI | With AI | Without AI | With AI |
| CA | 7.866 ± 2.599 | NA | NA | NA | NA | NA | NA | NA |
| GS | 7.468 ± 2.591 | NA | NA | NA | NA | NA | NA | NA |
| AI-only | NA | 7.366 ± 2.548 | NA | 0.489 | NA | 0.757 | NA | 91.03 |
| Rater 1 | 7.737 ± 3.205<br>$t = 2.58$ | 7.368 ± 2.562<br>$P = 0.010$ | 2.274<br>$t = -28.36$ | 0.494<br>$P < 0.001$ | 2.425<br>$t = -19.59$ | 0.770<br>$P < 0.001$ | 8.36<br>$\chi^2 = 229.33$ | 91.72<br>$P < 0.001$ |
| Rater 2 | 8.323 ± 3.144<br>$t = 6.98$ | 7.364 ± 2.550<br>$P < 0.001$ | 2.282<br>$t = -27.40$ | 0.488<br>$P < 0.001$ | 2.471<br>$t = -20.30$ | 0.752<br>$P < 0.001$ | 13.45<br>$\chi^2 = 215.35$ | 91.03<br>$P < 0.001$ |

CA, chronological age; GS, gold standard; BA, bone age; NA, not applicable; MAE, mean absolute error; RMSE, root mean square error; $\chi^2$ here denotes McNemar's $\chi^2$ test.

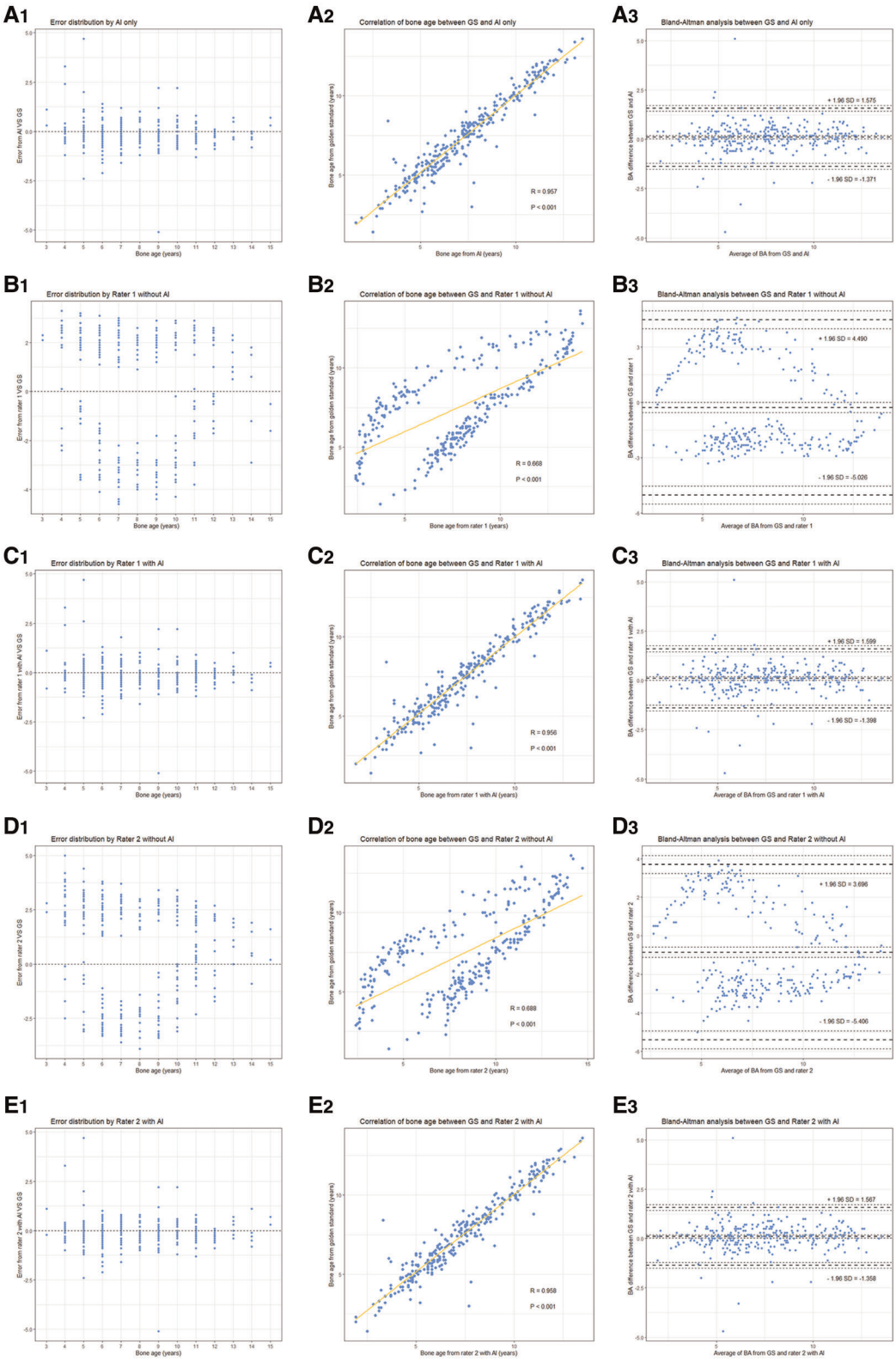


FIGURE 3
Performance of different raters in BA assessment. (left to right) Each column represents error distribution, correlation coefficient, and Bland-Altman analysis (95% LoA, limits of agreement), respectively. From top to bottom, i.e., (**A**–**E**) are the results performed by AI and the two junior raters (with or without AI assistance).

increased to over 91% (Table 2). Notably, the application of AI resulted in higher accuracy (observed in Rater 1) and lower MAE and RMSE (observed in Rater 2) when combined with the pediatric endocrinologists' interpretations as compared to AI alone. As illustrated in Figure 3, higher correlations (both had comparison $P < 0.001$) between the reference values and both readers ($r = 0.956$ and $r = 0.958$) could be observed upon the engagement of AI assistance. Bland–Altman plots revealed a decrease in the spread of ratings and decreased limits of agreement when paired with AI (Figure 3).

## Inter-rater and intra-rater variation with and without AI

ICCs were calculated to measure variations in inter-rater consistency both with and without AI assistance. The ICC between Rater 1 and Rater 2 without AI was 0.951 (95% CI, 0.830–0.978), which improved to 0.990 (95% CI, 0.987–0.992) with the assistance of AI. Overall, 62 radiographs were re-evaluated by the same raters both with and without AI. Repeated ANOVA showed significant variations (both with $P < 0.001$) between the initial and follow-up assessments of BA by the two junior pediatric endocrinologists. However, the significant differences disappeared (both have $P > 0.0125$) when the pediatric endocrinologists were assisted by AI (Table 3).

TABLE 3 Intra-rater effects in two times of bone age evaluation with and without AI.

| Raters | Bone age (mean ± SD) | | *F*-value | *P*-value |
|---|---|---|---|---|
| | First time | Second time | | |
| Rater 1 | 8.39 ± 3.77 | 7.45 ± 3.58 | 13.066 | 0.001* |
| Rater 1 with AI | 7.67 ± 3.28 | 7.45 ± 3.45 | 4.914 | 0.030 |
| Rater 2 | 8.73 ± 3.68 | 7.76 ± 3.27 | 13.070 | 0.001* |
| Rater 2 with AI | 7.63 ± 3.24 | 7.50 ± 3.30 | 1.760 | 0.190 |

Rater 1, Hou; Rater 2, Zhang.
*Denotes $P < 0.0125$ as Bonferroni correction was applied for four times of analysis using repeated ANOVA.

## Raters' effect in BA evaluation during the course of treatment

The mean reference BAs for the 52 children at baseline, 6-, 12-, 18-, and 24-months were 5.848 ± 2.302, 6.527 ± 2.385, 7.244 ± 2.329, 7.949 ± 2.458, and 8.411 ± 2.341 years, respectively. Without AI assistance, BAs as assessed by Rater 1 and Rater 2 both had gaps greater than 2 years (Table 4) from the GS at all 5 time points, yet such differences decreased sharply upon application of AI. There was a significant rater effect (Rater 2 only) in the BA values without AI during the course of treatment, although no obvious interactive effect (rater*time) was observed whether or not AI was deployed (Table 4). A detailed distribution of BA assessment by different raters in the longitudinal follow-up is displayed in Figure 4.

## Efficiency of rater-only and AI-assisted BAA

Overall, 290 radiographs were read by the 2 raters independently, which took 708 and 802 min to complete, respectively. When assisted by AI, their overall reading time decreased to 245 and 420 min, respectively, with the same number of images. Therefore, it could be said that AI helped to increase the pediatric endocrinologists' work efficiency by almost two-fold.

# Discussion

In this study, the performance of an AI-based BA evaluation system was assessed during the course of endocrine treatment among children with GHD. Based on the results, AI was proven to significantly improve both the accuracy and consistency within and between raters in BAA. To date, this is the first known study to explore the potential application of AI in BA evaluation in more practical and clinically common scenarios.

TABLE 4 Raters effect in bone age evaluation in the course of treatment from analysis using mixed linear model.

| Raters | △bone age (mean ± SD) | | | | | Rater's effect | | Time effect | | Raters*Time | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Baseline | 6 months | 12 months | 18 months | 24 months | *b* | *P* value | *b* | *P* value | *b* | *P* value |
| AI VS GS | 0.675 ± 1.103 | 0.558 ± 0.452 | 0.394 ± 0.337 | 0.463 ± 0.352 | 0.382 ± 0.287 | 0.122 | 0.330 | 0.677 | 0.000* | −0.069 | 0.076 |
| Rater1 VS GS | 2.327 ± 0.913 | 2.373 ± 0.787 | 2.308 ± 0.782 | 2.243 ± 0.863 | 2.239 ± 0.870 | 0.358 | 0.212 | 0.661 | 0.000* | −0.001 | 0.992 |
| Rater1 with AI VS GS | 0.758 ± 1.083 | 0.521 ± 0.523 | 0.398 ± 0.371 | 0.439 ± 0.334 | 0.386 ± 0.299 | 0.092 | 0.463 | 0.676 | 0.000* | −0.060 | 0.122 |
| Rater2 VS GS | 2.452 ± 0.985 | 2.242 ± 0.932 | 2.327 ± 0.983 | 2.280 ± 0.851 | 2.211 ± 1.047 | 0.838 | 0.002* | 0.661 | 0.000* | 0.046 | 0.590 |
| Rater2 with AI VS GS | 0.652 ± 1.070 | 0.571 ± 0.497 | 0.396 ± 0.360 | 0.455 ± 0.344 | 0.382 ± 0.290 | 0.077 | 0.539 | 0.675 | 0.000* | −0.053 | 0.170 |

GS, gold standard.
*Denotes $P < 0.01$ as Bonferroni correction was applied for five times of analysis using mixed linear model.

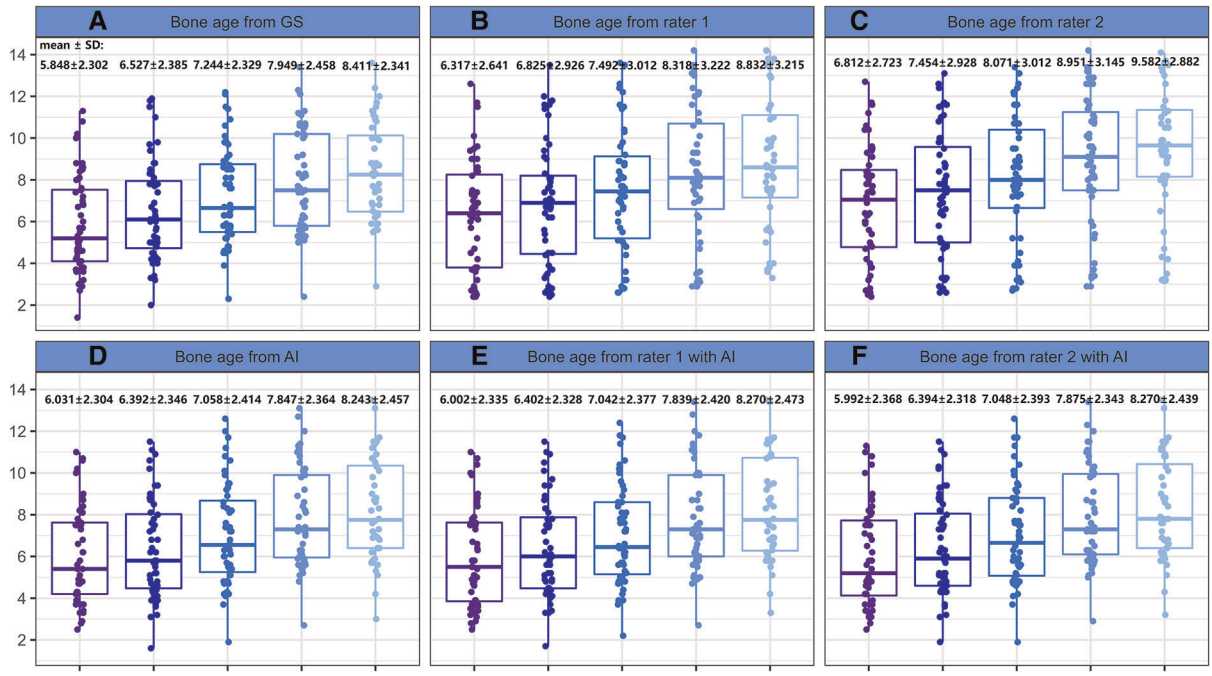


FIGURE 4
Boxplot of bone age as evaluated by different raters during the course of treatment. (**A**) Bone age from GS. (**B**) Bone age from rater 1. (**C**) Bone age from rater 2. (**D**) Bone age from AI. (**E**) Bone age from rater 1 with AI. (**F**) Bone age from rater 2 with AI.

This study's key finding implied that changes in BA values as assessed by the junior pediatric endocrinologists were significantly correlated with the raters during patient follow-up; even the BA rate showed inverse growth without AI. Delayed BA is a typical symptom of GHD, and BA catch-up is a common phenomenon of recombinant human GH therapy, which makes BAA useful in the evaluation of its therapeutic effect. However, the subtle BA changes during treatment can be difficult to detect by manual reading, especially in the absence of previous background information such as the diagnosis, results of previous ratings, or chronological age of the patient; as such, it is common to get a reverse increase in BAA (12). The determination of BA is commonly performed *via* visual comparison with the GP atlas or the TW3 method, so the outcome is prone to subjectivity. This occurs in part because when no perfect match exists in the reference material, readers must look for the reference image that exhibits the greatest similarity (10, 13), which is not conducive to the dynamic monitoring of BA and the therapeutic effects of treatment. The invention of AI assistance brings a possible, practical solution to this issue. As the results of this study suggest, BAA can be improved with the help of AI, which points to the benefits and significant clinical value of AI assistance in stable longitudinal BA monitoring. However, it should be noted that the results from this study were yielded by junior pediatric endocrinologists and excluded senior pediatricians and radiologists, so the results should be interpreted conservatively. Of note, clinical management of GHD treatment are based on height, height velocity and IGF levels, and BA recovery is only one of the referred factors. Hence, the process of BAA in follow-up, with or without AI, cannot replace the routine monitor of the indicators mentioned above.

This study demonstrated that the deployment of an AI assistance system decreased variations in inter-rater and intra-rater consistency. The results also built on recent data by Xi Wang et al., which demonstrated that an AI system based on CH05 BAA improved the performance of specialists with different levels of experience, thus increasing the ICC (14). The result also resonates with another study, which implied that an AI-assisted system could reduce variation in BAA by different raters, as well as the time required to read one radiograph (15). Improved consistency in BA evaluation would greatly benefit a variety of physicians and medical institutions in clinical practice, with the potential for increased precision in the diagnosis of endocrine disorders such as short stature (16), precocious puberty (17), and congenital adrenal hyperplasia (18).

Another goal of this study was to evaluate the efficiency and accuracy of BAA performed with the aid of AI. The results showed that not only could AI help to save time and increase the efficiency of BAA, which was a finding in common with previous studies (10, 14), but that the accuracy of both raters significantly improved after the assistance of an AI system

(8.36% vs. 91.72%, 13.45% vs. 91.03%). The results of this study agreed with that of previous research where an automatic BAA system was used to rate the BA of Iranian children, which yielded an accuracy within a 1-year range of 95.32% for radiographs of female patients, and 96.51% for radiographs of male patients, respectively (19). Another study demonstrated an accuracy of 84.6% by applying an AI system based on the GP method among Chinese children with abnormal growth and development (11). It is worth mentioning that the accuracy of a rater with AI assistance was higher than that of AI alone (91.72% vs. 91.03%). It has been proven that readers can achieve better BA accuracy with the assistance of AI compared to either readers alone or AI alone (15, 20). The difference in accuracy may be related to the ability of readers to identify skeletal deformity and malposition from hand radiographs as they determine the BA. Specifically, since BAA is a subjective process and is susceptible to clinical experience, young pediatric endocrinologists may have difficulty in determining every score for each ossification center confidently, swaying in two grades sometimes. While with the aid of AI, whose training algorithm involves a quantitative process with each pixel of the image, such ambiguity may decrease and a better performance achieved correspondingly. It is of worth noting that BAA is more commonly done at 6–12 monthly intervals in clinical practice, and here in this study we chose the shortest one to monitor. Since the use of AI when added to the interpretation by junior pediatric endocrinologists improved the MAE and RMSE over this short time, if clinically used at a greater time interval such as yearly as may be done in clinical practice, this could result in an improve in accuracy of interpretation.

## Limitations

The data used in this study was limited to a single center within a single region, thus involving a relatively small data volume. The included data were mainly obtained from children between the ages of 3–13, with limited data taken from other age groups. This study only compared the values among junior pediatric endocrinologists under the absence and presence of AI assistance. In the future, the involvement of senior pediatricians and radiologists would be helpful in further elucidating the practicality and clinical value of AI assistance in BAA.

## Conclusion

In conclusion, the AI system in this study, which was constructed based on the CH05 BAA standard, was found to exhibit a high degree of accuracy with only slight deviation in the diagnosis and follow-up of GHD. With the increase of available sample data and further development of deep learning methods, the accuracy and efficiency of the automatic BAA can continue to be further expanded upon and improved. A future multi-center study will make BAA even more clinically adaptable.

## 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 study was approved by the Ethics Committee of Sun Yat-sen Memorial Hospital.

## Author contributions

LL and ZM: contributed to the conception and design of the study. LZ and JC: contributed to the data analysis and preparation of the manuscript. LH and ZL: contributed to data collection and study conduct. YX, SH, and HO: contributed to case follow-up. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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

## Publisher's note

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# Impact of glucose metabolism on the developing brain

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Glucose is the most important substrate for proper brain functioning and development, with an increased glucose consumption in relation to the need of creating new brain structures and connections. Therefore, alterations in glucose homeostasis will inevitably be associated with changes in the development of the Nervous System. Several studies demonstrated how the alteration of glucose homeostasis - both hyper and hypoglycemia- may interfere with the development of brain structures and cognitivity, including deficits in intelligence quotient, anomalies in learning and memory, as well as differences in the executive functions. Importantly, differences in brain structure and functionality were found after a single episode of diabetic ketoacidosis suggesting the importance of glycemic control and stressing the need of screening programs for type 1 diabetes to protect children from this dramatic condition. The exciting progresses of the neuroimaging techniques such as diffusion tensor imaging, has helped to improve the understanding of the effects, outcomes and mechanisms underlying brain changes following dysglycemia, and will lead to more insights on the physio-pathological mechanisms and related neurological consequences about hyper and hypoglycemia.

KEYWORDS

**glucose metabolism, hyperglycemia, hypoglycemia, type 1 diabetes, brain**

## 1 Introduction

The mechanisms and processes of brain maturation are among the most fascinating aspects of human physiology and anatomy. Despite brain remodeling occurs continuously throughout life, the first and most relevant stages of its maturation take place in the first two decades.

The newborn's brain is about a quarter or a third of the size of the adult's brain and grows and specializes according to a precise genetic program (1, 2). Because of the interaction with the external environment and the experiences acquired, the dendritic ramifications increase enormously, as well as the synaptic connections that undergo remodeling and "pruning" processes throughout all life (3). The massive structural,

volumetric and connectomic changes that occur in the first years of life require adequate energy substrates. Therefore, a correct supply of substrates and a dynamic brain metabolism is fundamental for these changes.

We hereby evaluate the cerebral changes of glucose metabolism in children and examine the impact of hypoglycemia and hyperglycemia on the developing brain.

# 2 Glucose metabolism in the developing brain

The human brain is the most metabolically and energetically expensive tissue within all organs, and glucose is the predominant organic fuel used in all animal species, including humans (4, 5).

The adult human brain consumes approximately 20-25% of the total amount of glucose used by the body, whereas the growing brain consumes an even greater amount (6, 7), with some estimates suggesting that the glucose consumption in the infant brain constitutes more than 40% of the body's basal metabolic rate (8).

## 2.1 Glucose uptake

While the importance of glucose for proper brain function has been a long-established concept, the exact mechanism by which glucose is able to reach brain tissue is a much more recent discovery. Glucose, as a hydrophilic and polar compound, is unable to spontaneously diffuse across the endothelial membrane. For this reason, the conformation of the blood-brain barrier, with tight junctions between endothelial cells, requires specific transcellular glucose transporters from the blood to the brain. This family of glucose transporters (GLUT) is responsible for the entry of glucose into cells, mediating more than 95% of glucose transport to nervous tissues (9, 10). Several members of this family of transporters have been found in the brain: GLUT 1 to 5 and, more recently, GLUT6 (previously referred to as GLUT9), GLUT8, GLUT10 and GLUTX1 (11). Among these, the most important ones are GLUT 1 and GLUT 3 (12, 13). These transporters exhibit regional heterogeneity in the brain tissue and their expression is regulated at transcriptional, post-transcriptional and post-translational levels by external stimuli, including hypoxia, insulin, hyperglycemia and hypoglycemia, and increased brain glucose demand (5, 9). However, glucose uptake in neurons is independent from the action of insulin, relying on the extracellular concentration of glucose; this exposes the brain to a higher chance of damage compared to other human cells (14).

## 2.2 Brain glucose consumption

Approximately 30% of circulating glucose levels are located in brain extracellular fluid, with about 20-30 minutes of stabilization time during periods of glycemia's alterations (15, 16). Not all glucose is metabolized immediately, but a great part is stocked in the form of glycogen and held within astrocytes (15). Most of the energy produced from glucose metabolism (70%) is used for neuronal signal transmission functions such as action potential, calcium activities, synaptic transmission, and glutamate cycling; the remaining part is involved in non-signaling activities, like axonal transport, resting potential, and cytoskeleton remodeling. Furthermore, glucose metabolism provides the carbon used for nucleic acids, fatty acids and amino acids synthesis, and produces metabolites that are involved in the regulation of inflammatory and redox reactions (17–20).

Brain glucose consumption can be quantified using the Cerebral Metabolic Rate for Glucose (CMRG), measured by knowing the cerebral blood flow and the arteriovenous glucose difference in the brain, or estimated using fluorodeoxyglucose positron emission tomography (FDG- PET) (5). Because of the invasive aspects of the procedure and the ethical issues in conducting radiodiagnostic investigations in children, there are few studies performed on healthy children: most of them are conducted in young patients with epilepsy, with suspicion of hypoxic-ischemic damage or neonatal hypoglycemia, with autism or when malignancy is suspected.

One of the first studies about the difference in brain metabolism between children and adults was published more than 60 years ago (21). This study showed that the CMRG was significantly higher in children compared to healthy young adults. These findings have been confirmed by a subsequent study on 29 healthy children aged between 5 days and 15 years of age, which investigated for transient and sequelae-free neurological events with FDG-PET (22). The study showed that not only the use of glucose changes with age, but also that, depending on the age of the child, glucose is used differently between the areas of the brain. As also showed in animal studies (23–25), during different stages of development cerebral structures with high CMRG determine the predominant behavioral pattern at the particular evolutionary stage (Table 1). Moreover, whole brain CMRG was found to be closely correlated with post-conception age and postnatal age, being lower in the preterm infant compared to children aged 50-60 postconceptional weeks (26).

In the first two years of life, the CMRG is comparable to the one of a young adult, but with different anatomical patterns depending on the months of life. In term newborns, the most metabolically active areas are the thalamus, the cerebellum, the

TABLE 1 Fluorodeoxyglucose – positron emission tomography (FDG-PET) patterns according to age.

| Age | FDG-PET pattern | Neurological features |
|---|---|---|
| Newborns | Subcortical brain structure | Intrinsic reflexes |
| 3 months | Parietal cortex<br>Primary visual cortex<br>Cerebellar hemisphere | Visual-spatial and<br>visual-motor integration |
| 8 months | Dorsolateral and Frontal-occipital cortex | Greater interaction with the environment |
| >2 years | Global increased glucose consumption | |
| 3-5 years | Twice the value compared to adults | |

sensorimotor cortex and the basal ganglia at the expense of the visual cortical regions and the frontal cortex (27–29). In preterm infants, compared to term infants, despite the increased metabolic activity persists in the subcortical regions, the cortical areas are metabolically less active and the FDG-PET images show a finer signal (27). Furthermore, brain development is associated with both significant linear and non-linear changes in regional glucose metabolism in various cortical and subcortical structures from birth to adulthood (30). These changes may also correlate with significant modifications in neurometabolic connections involving the fronto-thalamic, fronto-cerebellar and fronto-hippocampal networks, representing a metabolic correlation between age-dependent effects on sensory, motor, and high-level cognitive functional networks (30).

The neonatal behavior, which is characterized by intrinsic reflexes, is mainly dominated by the activity of the subcortical brain structure, whereas at about 3 months of life afinalistic movements leave space for more coordinated movements that require visual-spatial and visual-motor integration. This is reflected by an increase in the CMRG at the level of the parietal cortex, the primary visual cortex and the cerebellar hemispheres. At about 8 months of life, there is an increase in CMRG at the level of the dorsolateral and frontal occipital cortex, which reflects the greater interaction that the child has with the environment in this period of life. Subsequently, from 8 months to 2 years of life, the absolute CMRG for many structures was similar to that found in young adults. Starting from the second year of life there is an increase in the CMRG which reaches twice the value of the one recorded in adults at around 3-5 years of life, to later progressively decrease between 9 and 15 years (22).

These data were confirmed by more recent studies that extended the cohorts to preterm infants and to a larger group of patients (26–32).

Some authors found that brain metabolism, calculated through FDG-PET acquisitions, was significantly different between males and females aged between 15 and 17 years, with an increase in FDG uptake recorded in females (33). This increase in metabolic activity was consistent with the finding reported in previous volumetric studies, that women mature earlier than men (1, 2).

## 2.3 Aerobic glycolysis

Along with the increased consumption of glucose, some studies showed that in childhood there is also an increased use of oxygen in the brain (34).

The metabolic pathway of oxidative phosphorylation is the main supplier of adenosine-50-triphosphate (ATP), producing up to 36 molecules of ATP per glucose molecule, through a series of phases like glycolysis, the citric acid cycle, and the electron transport chain (35, 36). Traditionally, the oxidative metabolism pathways are preferred, unless perturbation of oxygen supply (such as hypoxia/anoxia) or mitochondria impairment occurs (36). However, recent data suggest that in the brain, glycolysis also happens under sufficient oxygen conditions (35, 36). This phenomenon, called *aerobic glycolysis* (AG), it is not exclusive to brain cells: cancer cells, which are characterized by rapid and uncontrolled proliferation, shift glucose consumption towards AG in order to support the biosynthetic reaction needed for cellular growth (7). Moreover, AG is the primary pathway during proliferation of many fast-growing unicellular organisms, regardless of oxygen availability (35). Thus, it is not surprising that in a developing brain, aerobic metabolism is favored.

During early post-natal and childhood, the AG increases enormously accounting for about a third of the total glucose consumption at about 5 years of life (7). This phenomenon could be one of the main reasons for a greater glucose demand - hence, causing an increase in CMRG; AG seems to be necessary for the development of new nerve structures such as synaptic formations, axonal elongation and myelination (36).

# 3 The contribution of radiodiagnostics

The study of brain glucose metabolism has undergone considerable progress over the years thanks to the use of less

invasive techniques that are more easily applicable to the pediatric population.

The first studies on brain glucose metabolism were based on the calculation of the CMRG, by estimating the cerebral blood flow and the difference in glucose measured in the arteries and veins of the brain (5). Subsequently, with the use of the FDG-PET it was possible to estimate glucose metabolism by analyzing the concentration of the radiopharmaceutical at the tissue level and calculating the relative Standardized uptake Volume (SUV) (37, 38). Indeed, SUV values provide an alternative for estimating cerebral glucose uptake by showing a good correlation with CMRG values (39).

Today more innovative and less invasive techniques are able to analyze brain metabolism and they have also been used in the pediatric field. Proton Magnetic Resonance Spectroscopy (1HMRS) is an advanced imaging technique used to detect information on the biochemical composition of the tissues analyzed in a non-invasive way (40, 41). 1HMRS processes the signals from the hydrogen protons to determine the relative concentrations of tissue metabolites including choline, N-acetylaspartate, lipids, glutamine, glutamate and glucose (40, 42). Clinical uses in pediatrics include the diagnosis of brain tumors, neonatal disorders such as hypoxic-ischemic encephalopathy, inherited metabolic diseases, traumatic brain injuries, demyelinating conditions and infectious brain injuries. However, routine implementation of 1HMRS is hampered by the lack of measures of control, acquisition protocols and standardized analysis techniques and the lack of a reference spectrum appropriate for the age of the subject, not yet fully available (40, 43).

An alternative approach is given by the spectroscopic analysis with Nuclear Magnetic Resonance (NMR) after infusion of a non-radioactive substance, the 1- (13)C glucose (44, 45). This compound crosses the blood-brain barrier allowing to map the anatomical distribution and quantify the cerebral concentration of glucose (44). Despite the potential applications in the study of abnormalities of brain glucose consumption, the role of 13C NMR in clinical practice is still a subject of speculation (45).

As for the study of cerebral metabolism, many advances have also been made for the morpho-structural study of the brain. Conventional MRI is useful to assess whole brain measurement and differences between white and gray matter. However, few studies managed to explore the differences during development (14). For example, some authors compared structural MRI findings in children with and without diabetes during a 18 months period, demonstrating a significant reduction in growth of cortical gray matter volume, cortical surface area and white matter volume throughout the cortex and cerebellum. In addition, the authors suggested that fluctuating glucose levels in diabetic patients may also be associated with corresponding fluctuations in brain volume, since they found a negative correlation between change in glycemia levels and change in gray and white matter volumes in these children at the time of the scan across longitudinal time points (46).

Over the last decade, a new type of magnetic resonance imaging called diffusion tensor imaging (DTI) has been developed, which is uniquely suited to study the white matter microstructure (47). DTI measures the magnitude and directionality of water diffusion in tissues distinguishing between isotropic diffusion and anisotropy diffusion (47, 48). Among its applications, this technique has been used to evaluate the difference in brain structure between children with DT1 and age-matched controls, which found a significant change in fractional anisotropy and apparent diffusion coefficient in widespread regions of the brain, which may represent early features of injury to myelinated fibers and/or axon degeneration (49).

# 4 The impact of abnormalities of glucose homeostasis on the developing brain

Childhood and adolescence are the most significant times of neurodevelopmental changes (50). Alterations in glucose metabolism during these periods can have a long-term impact on brain development and cognitive function.

Human data and experimental data on animals suggest that both hypoglycemia and hyperglycemia, depending on age and severity, can alter brain structures and cognitive functions (51–57). In fact, it is proven by many *in vitro* studies that stable glucose levels are fundamental for neuronal functionality and activity (15, 18–20). Periods of abnormal glucose levels impact negatively neuronal activity and survival, as well as situations of rapid glucose fluctuation can cause neuronal injury (15).

## 4.1 Effects of hyperglycemia

As previously mentioned, the uptake of glucose is dependent from its extracellular concentration, exposing neurons to damage when exposed to hyperglycemia (14). Brain cell injury is induced by different mechanism. First, the increase in glucose levels induces an increase in the permeability of the blood brain barrier, which allows the entry of glucose and other substances capable of damaging the central nervous system (58, 59). Glucose can react with intra and extracellular compounds, triggering the production of reactive oxygen species, subsequently leading to oxidative stress. This will later cause a modification of cellular molecules, which will therefore lose their functionality leading to mitochondrial dysfunction and cellular damage (14).

If this event occurs early, the resulting alterations in the brain organization could make the brain more susceptible to future events in chronic conditions such as type 1 diabetes

(T1D). Indeed, chronic hyperglycemia can lead to the formation of end products of advanced glycation, increase oxidative stress and even neurodegradation (56, 60, 61).

Similarly to streptozotocin-induced diabetes animal models, which show *in-vivo* degenerative changes of neurons and glia, disarrangement of myelin sheaths and reduced myelin content, glucose variability may damage developing neurons in children (53, 56, 57, 61). In addition, hyperglycemia can induce changes in the composition of brain sphingolipids (ceramides and sphingomyelin) causing membrane rearrangements in some cell populations (62).

### 4.1.1 The relevance of T1D

The most important pathology determining alterations of glucose homeostasis in the pediatric population is T1D (63). The negative impact of dysglycemia on brain structure and function has been extensively studied in children and adolescents with T1D, leading to the description of common findings of volumetric and structural brain alterations (64) (Table 2).

The major neuropsychological sequelae are observed in children with onset of diabetes between the first 5-7 years of life (57, 63–65), with cognitive and structural deficits observed shortly after diagnosis (65, 66). Indeed, the phase preceding the diagnosis of diabetes is characterized by a long period of uncontrolled hyperglycemia and consequent neurotoxicity, which represents a crucial moment in the genesis of the brain damage (58).

#### 4.1.1.1 Radiodiagnostics in T1D

Brain imaging studies have shown that T1D is associated with total and regional reduction in gray matter and white matter volumes (44, 66–71). This reduction is proportional to the time of onset of diabetes, with a more drastic change in brain parenchyma in patients who had an earlier onset of T1D compared to the later-onset T1D group (72). Regional differences may be explained by a higher glucose demand required by certain brain regions, such as the frontal and temporal lobes (65). Indeed, it was frequently recognized a decreased mean gray matter in the frontal precentral and temporal regions, but also in the thalamus and insular cortex (70, 73). A study evaluated 144 T1D children aged between 4 and 10 years and 72 healthy children, through two MRI acquisitions performed 18 months apart: children with diabetes had less cortical gray matter growth than controls, with significant localized differences in the left precuneus extending to the left parietal and posterior-occipital region and the right temporal, frontal and parietal lobes. Similarly to what was found for the gray matter, there were also widespread differences in white matter, with slower growth in T1Ds vs. controls, including the splenium of the corpus callosum, bilateral superior-parietal lobe, bilateral anterior forceps, and inferior-frontal fasciculus, with the main findings identified in the right anterior-frontal lobe (74). A subsequent analysis of the same images also showed that differences in brain substance growth in diabetic patients were most pronounced in the younger tiers among the age range (46).

Volumetric white matter abnormalities in children with T1D have also been correlated with impaired connectivity and cerebral microstructures (75–77). Likewise, some authors demonstrated lower axial diffusivity in children with T1D compared to age-matched controls, and related these differences to a higher average exposure to hyperglycemia, suggesting again a disruption of the physiological development of brain myelination and the importance of achieving an optimal glucose control in these patients (78).

Other authors investigated white matter microstructures abnormalities in T1D patients and healthy controls between 9 and 22 years of age, using DTI (77); they demonstrated that in children with T1D the superior parietal lobule had altered DTI parameters compared with controls correlating this alteration to axonal injury (77, 79). Anomalies of axial diffusivity were also found at the level of the temporal area (75, 76). In addition, a significant correlation emerged between the anomaly of axial diffusivity and the values of glycated hemoglobin recorded at the time of acquisition of the radiodiagnostic images (75).

Relevant data on brain alterations resulting from dysglycemia have recently been brought to light by a study carried out through the acquisition of spectrometric images in children with T1D, a decrease in choline and N-acetylaspartate at the level of the pons was found suggesting a neuronal loss or function impairment in that area, with changes in membrane lipids and/or a decreased membrane turnover (80). A reduction in N-acetylaspartate was reported by human and animal studies and, as a marker of neuronal density, remarking the link between chronic hyperglycemia, demyelination and loss of neurons (53,

TABLE 2 Neuro-radiological findings in pediatric type 1 diabetes (T1D).

| CONVENTIONAL MRI | DTI | SPECTROMETRY | FUNCTIONAL MRI |
|---|---|---|---|
| Total and regional reduction in gray and white matter volumes, proportional to the onset of T1D | Widespread fractional anisotropy reduction and lower axial diffusivity in correlation with glycated hemoglobin values<br>Higher fractional anisotropy in children with lower exposure to hyperglycemia | Lower mean N-acetyl aspartate and higher mean myoinositol and choline, suggesting a neuronal loss or function impairment | Hyperactivation of task-positive regions underlying attentional and executive control, to counteract T1D-associated abnormalities in brain structure and facilitate cognitive and behavioral function |

73). An increase of myoinositol and choline was also reported in T1D patients, demonstrating an alteration of osmolarity, demyelination and glial hypoxic damage (81).

#### 4.1.1.2 T1D and cognitivity

The impact of dysglycemia on cognition and school performance in children has been the topic of many studies, but it is still a subject of considerable controversies (82). Several studies have found worse outcomes in different cognitive domains in children with early onset diabetes. Ferguson et al. compared MRI brain structures and cognitive ability in a group of young adults with long-duration T1D diagnosed during childhood or adolescence (72). The study reported greater lateral ventricular volumes, more prevalent ventricular atrophy and poorer intellectual and information processing abilities in the early-onset T1D group (72). Similar neurological implications were also reported in children with diabetes and early-onset severe hypoglycemia: these patients were found to have a poorer cognitive performance compared to those with late-onset severe hypoglycemia (83).

Changes on intelligence quotient (73), anomalies in learning and memory (81, 84, 85) and differences in the executive functions (81, 86) have also been reported (Table 3). Some authors performed a cognitive study on children with diabetes and on healthy controls, observing cognitive differences in children with diabetes in the areas of intellectual ability and executive functions (66). However, these results were not confirmed in a subsequent re-evaluation performed after 18 months (96). Similarly, equivalent cognitive and behavioral performance were recorded among 93 T1D children and 57 non-diabetic controls. The study analyzed functional MRI of children performing an executive function paradigm, and found an increased activation in executive control regions, as well as reduced suppression in the posterior node of the default mode network. Specifically, worse suppression of the default mode network in children with T1D was associated with hyperactivation of task-positive regions underlying attentional and executive control, suggesting that this activation of executive control networks may transiently counteract T1D-associated abnormalities in brain structure in order to facilitate normative cognitive and behavioral function (63).

Another important aspect to consider in the set of T1D, is the relationship between cognitive deficits and diabetic ketoacidosis (DKA). DKA is the most common acute cause of morbidity and mortality in children with T1D, with neurological consequences that present acutely with the development of

TABLE 3 Neurocognitive sequelae resulting from the main alterations of glucose metabolism in children with type 1 diabetes.

| Clinical Outcomes | T1D | Early Onset T1D | DKA | Poor Glycemic Control | |
|---|---|---|---|---|---|
| | | | | Hypoglycemia | Hyperglycemia |
| **Global intelligence** | -Lower intelligence (crystallized and fluid) (84)<br>-Lower verbal IQ scores in boys (87) | -Lower performance IQ (88) | - Lower performance IQ in moderate/severe DKA group compared with the none/mild DKA group (89) | -Lower verbal IQ (88)<br>-General intelligence deficit in severe hypoglycemia (52) | -Low general cognitive abilities (90) |
| **Learning and Memory** | -Poorer working memory (84) | -Poorer sustained and divided attention and poorer new learning (81)<br>- Lower verbal and visual learning and memory ability in early onset vs late onset T1D (84) | Lower rates of accurate memory (91)<br>Poorer delayed memory recall and poorer sustained and divided attention (15) | -Poorer working memory, and non-verbal processing speed (81) | - Poorer working memory (81)<br>-Low receptive language function (90)<br>- Poorer long-delay spatial memory at the time of assessment of T1D (92) |
| **Executive functions** | - Lower psychomotor efficiency (84)<br>-Lower attention and executive function (84) | | | - Poorer verbal ability (81)<br>-Verbal fluency/ language deficits in severe hypoglycemia (52) | - Slow fine motor speed (90)<br>-Reduced spelling performance (93) |
| **Neuropsychiatric disorders** | -Increased risk in suicide attempts and in most categories of psychiatric disorders (94)<br>-Increased risk of neurodevelopment disorders (95) | | | | -Higher risk of ADHD, ASD (95) |

ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; DKA, diabetic ketoacidosis; IQ, intelligence quotient; T1D, type 1 diabetes.

cerebral edema. Therefore, several studies questioned the long-term neurological effects of DKA on children's brain, reporting an impact on brain morphology and functionality, especially in memory function (15, 91, 92). Importantly, a study on a cohort of children with T1D found that, compared with patients without or with mild DKA, patients with moderate/severe DKA have lower cognitive scores and altered brain growth after a single episode of DKA (89).

Several studies have also suggested a link between childhood T1D and increased risk of neurodevelopmental disorders, including attention-deficit/hyperactivity disorder (ADHD), autism spectrum disorders (ASD) and intellectual disability (94, 95, 97). A recent large population-based cohort study showed that individuals with T1D bear a significantly higher risk of developing any neurodevelopmental disorders (Hazard Ratio 1.3), ADHD (Hazard Ratio 1.29) and ASD (Hazard Ratio 1.31) compared with matched reference individuals. This risk also increased with higher mean HbA1C levels, demonstrating that poor glycemic control, assessed using time-varying HbA1C, was an independent risk factor for subsequent neurodevelopmental disorders (95).

Long-term outcomes may also differ by gender. In a study conducted on 64 children between 7 and 16 years old, there was a significant decline in performance by age 7 and in the verbal intelligence quotient between the age of 7 and 16. This was exclusively found in boys with onset of diabetes before 6 years old, but not in those with a later onset and not in diabetic girls (87). Furthermore, diabetic children have been found to read more slowly, to make more time-consuming errors than control in both genders and to have less capacity in the use of spatial information and in language skills in males and females respectively.

However, despite the negative impact of the alteration of glucose homeostasis on the developing brain, to date it is still not possible to fully determine what are the possible cognitive consequences (85). Future studies are needed to clarify the extent and long-term effects of these anomalies.

### 4.1.2 Neonatal hyperglycemia

Birth causes the cessation of the continuous supply of glucose from the mother; therefore the newborn must use his or her own stored substrates, activating glucose regulation mechanisms in the first minutes of life. In newborns glucose levels decrease at 1-2 hours of age and subsequently increase over the first 18 hours. Glycemia remains stable for the next 48 hours and subsequently increase during the third day of life after birth (98).

However, numerous mechanisms can interfere with this balance, causing hyper or hypoglycemia in the perinatal period. High levels of blood glucose are most commonly reported within the first 3 to 5 days of life, as a consequence of limited insulin secretion capacity, increased counter-regulatory hormones, sepsis, parenteral glucose and medications' administration (e.g. steroids) (99). Moreover, due to environmental (drugs, parental glucose infusion, sepsis, intrauterine growth restriction) and intrinsic factors (e.g. alteration of hormonal regulation with reduced insulin production and reduced suppression in hepatic glucose production), preterm infants have an increased risk of hyperglycemia (99–102), which is inversely correlated to gestational age (103).

However, hyperglycemia may also occur in term infants, especially those treated with therapeutic hypothermia for hypoxic-ischemic encephalopathy (HIE) and, less commonly, in term infants with neonatal diabetes (104).

Among the main neurologic effects of perinatal hyperglycemia the most frequently encountered short-term complications are white matter image changes and intraventricular hemorrhages (98). In a study of term infants with moderate-to-severe HIE who received therapeutic hypothermia, it was found that infants with hyperglycemia have a higher likelihood of having basal ganglia damage or a global pattern of injury compared to infants with normal blood glucose values, but also a higher frequency of normal MRI compared to the hypoglycemic group (105).

In preterm children early exposure to high glucose levels is associated with white matter reduction on imaging, a greater risk of developing intraventricular hemorrhage and small head circumference (106–108).

Evidence of long-term neurodevelopmental outcomes in term infants is lacking and present conflicting results. In term infants, exposure to hyperglycemia in the first hours of life is correlated with an increased risk of moderate to severe cerebral palsy, poor gross motor outcomes (104, 109) and seizures (110). However, in another study the occurrence of hyperglycemia was not associated with an adverse outcome (111).

Similarly, in preterm babies the relationship between neonatal hyperglycemia and neurodevelopmental outcome remains controversial. Moreover, close monitoring of neonatal hyperglycemia does not improve long-term neurodevelopmental outcomes (112), whereas in other studies neurodevelopment impairment, abnormal behavior development and abnormal executive function have been identified (106, 112).

Another cause of hyperglycemia in neonates – although less common – is neonatal diabetes mellitus (NDM). Differently from classic T1D, which onset is the result of the interplay between a genetic predisposition and the environment, NDM is most often caused by monogenic deficits that involve cellular channels (e.g. ATP-sensitive potassium channel, ABCC8) or alteration in the insulin gene (INS). NDM occurs in approximately 1 in 90,000-160,000 live births (113) and should be considered in infants with insulin dependent hyperglycemia, without an alternative etiology, for longer than seven to ten days (114, 115).

Neurodevelopmental problems are frequently reported in NDM, although it is not easy to tell if they are the consequence of

the genetic abnormalities or of the glycemic excursions (116). Global developmental delay and impairment in many areas of functioning are among the neurological symptoms reportedly related to NDM that are caused by the KCNJ11 mutation (116), whereas mutations in IER3IP3 are associated with severe epileptic encephalopathy, microcephalia and seizures (117). Interestingly, treatment with sulfonylurea was found to improve the neurological outcome of patients with NDM, supporting the notion that an early diagnosis of NDM may improve the outcome of these children (118).

In a neuroimaging study conducted on children with persistent NDM, different anomalies from those reported in children with T1D, emerged including multiple punctate with matter hyperintensities on the T2 and FLAIR sequences and hyperintensities in the raphe pontes nucleus (114). However, studies about the neurological outcome of children with T1D compared to the ones with NDM are lacking; thus, it is not known if the differences are linked to the causative genetic mutations or to the early exposure to hyperglycemia.

## 4.2 Effects of hypoglycemia

Hypoglycemia is the form of dysglycemia most readily associated with neuronal insult. Indeed, severe hypoglycemia can cause altered consciousness, progressing to seizures or coma and eventually death. Although there is not a universal defined threshold, progressive cognitive dysfunction seems to occur below a blood glucose level of 3.0 to 3.5 mmol/L (119).

The biochemical mechanisms through which severe hypoglycemia cause neuronal damage are not completely known. It may trigger the synaptic release of excessive glutamate causing intracellular calcium toxicity and excitotoxic cellular damage (120); it may cause the overstimulation in addition of the N-Methyl-D-aspartate receptors, resulting in excitotoxicity first and cell damage later (14). In the set of energy restriction (e.g. fasting, prolonged exercise), the brain cells start using alternative fuels for their metabolism like ketone bodies, which can provide up to 60% of metabolic requirements (119, 121). Some animal studies have demonstrated that brain can both benefit and be injured from the use of these metabolites. Indeed, ketones may reduce infarction and edema in response to hypoxia induced injury when exogenously administered, but it is also documented that in situation of endogenic ketoacidosis they seem to reduce cerebral blood flow by increasing levels of vascular permeability factors and vasoconstrictors as endothelin-1 (15, 91).

Not only extremely high or low levels of glycemia are dangerous, but also rapid fluctuations have a negative impact on cells homeostasis. In studies conducted on neural cell culture models it has been demonstrated that approximately 6 hours fluctuations in glucose levels may affect cellular metabolism, through a decrease in mitochondrial activity and the activation of intrinsic apoptotic pathways (19, 119).

Nevertheless, there are currently conflicting opinions about the role of hypoglycemia.

Some reports identify hypoglycemia as a risk factor for mild cognitive dysfunction and structural anomalies (81, 122, 123), while other studies, also performed on animal models, have not found statistically significant results in this regard (70, 96, 124). MRI studies have described how hypoglycemia may compromise normal hippocampus development, with findings of gliosis and reactive neurogenesis (125).

A further aspect to consider is the impact of the frequency of hypoglycemic episodes. A meta-analysis studied the effect of recurrent severe hypoglycemia on cognitive performance in children with T1D and reported a slight but significant decrease in cognitive performance in T1D children with episodes of severe hypoglycemia compared with those without such episodes (52). In particular, statistically significant differences were found on four cognitive domains: intelligence, learning, memory and verbal fluency (52). Neuroimaging studies support these findings, noticing that severe hypoglycemia preferentially targets neurons in the cerebral cortex, particularly in the medial temporal region, including the hippocampus (involved in memory functions), basal ganglia and brainstem. The neuroimaging studies reported in a meta-analysis also documented a reduction in gray matter volume at the left temporal-occipital junction in diabetic children with one or more severe hypoglycemia episodes (68), and a relatively lesser gray matter density in children with a history of severe hypoglycemia (126), with a high prevalence of damage to the hippocampus and the cornu ammonis (52).

### 4.2.1 Congenital hyperinsulinism

In childhood, the most common cause of hypoglycemia is represented by Congenital Hyperinsulinism (CH), a heterogeneous congenital disorder characterized by a dysregulation in insulin secretion which results in random hypoglycemia associated with low or normal ketones and no metabolic acidosis (127). CH is frequent in neonatal age (typically within the first week of life), but less common in infants, toddlers, and older children (127, 128). From a physio-pathological point of view, in CH there is a uninhibited insulin secretion due to an altered plasma glucose-insulin feedback regulation, which causes unpredictable and severe hypoglycemia, but also represses lipolysis, leading to a reduction of ketones' formation, the alternative brain fuels to maintain neuronal function (128).

CH usually presents with persistent and unpredictable episodes of hypoketotic hypoglycemia, which are detrimental to the vulnerable neonatal brain and are associated with adverse neurodevelopment (128). A study assessed that a third to half of children with CH present with adverse neurodevelopment

regardless of the type of CH (focal/diffuse or transient/permanent) The consequences of perinatal hypoglycemia can vary from MRI-detectable extensive bilateral areas of cystic encephalomalacia in the occipital-parietal area and posterior temporal lobes, to more subtle effects like mild motor delay, definable only by formal cognitive assessment (128). Moreover, brain insult topography is related to the age of hypoglycemic manifestations (129, 130) as demonstrated by the increased risk of pyramidal tract damage in newborns that present hypoglycemia in the first day of life (131).

Not all children have severe and persistent forms of the disease (128), but it is important to early identify them because early-life hypoglycemia has a negative impact on cognitive function of these patients, especially for the ones with an early onset. Indeed, neurodevelopmental abnormalities (e.g. motor and speech impairment) due to delayed diagnosis and suboptimal management are reported in 25%–50% of CH cases (129, 132).

### 4.2.2 Neonatal hypoglycemia

Hypoglycemia is one of the most common disorders in neonates, occurring in as many as 19% of infants overall (133, 134).

Numerous risk factors, both maternal and fetal, have been identified for neonatal hypoglycemia (NH). The causes of NH can be briefly divided into: a) increased glucose utilization in sick or stressed infants (e.g. neonates with perinatal hypoxia-ischemia or congenital heart diseases); b) hyperinsulinemia due to maternal gestational diabetes, obesity or due to birth asphyxia, placental insufficiency or post maturity; c) inadequate substrate stores in preterm or Intra Uterine Growth Restriction or Small For Gestational Age infants; d) iatrogenic causes, such as hypothermia, malposition of umbilical catheters; e) hormonal deficiencies in infants with hypopituitarism, hypothyroidism, adrenal insufficiency; f) congenital disorders, like Beckwith-Wiedmann syndrome, Down syndrome, Turner syndrome; g) defects of glucose transporter (98). However, most causes of NH are multifactorial (134).

In conditions of hypoglycemia, the newborns' brain can use alternative energy substrates such as ketone bodies, amino acids and lactate; it also increases cerebral flow, improving the transport of substrates for glycogenolysis, and reduces neuronal activity (135, 136). Anyway, these alternative fuel sources may not be available in preterm or Small For Gestational Ages infants, or may be unable to be used due to perinatal factors such as hypoxia, seizures or pathologic jaundice (137). Therefore, the energy failure caused by consuming or not using alternative substrates may result in cerebral injury (136).

Long term sequelae can occur within a wide range of low serum glucose values, and even transient moderate hypoglycemia may result in neurological damage (138). Cerebral injury caused by NH includes cortical neuronal injury, ischemic stroke, parenchymal hemorrhage and white matter injury (134, 139–141). Since the first neuroimaging studies, a disproportionate involvement of the occipital and parietal lobes emerged (142). This involvement was then confirmed by subsequent studies which also identified abnormal signals in the deep gray matter structures of the thalamus and basal ganglia (26, 143) and diffusion restriction in the parietooccipital areas, underlying white matter, and corpus callosum (144).

The reasons why the occipital cortex is so sensitive to hypoglycemia in newborn period are not clear. Animal studies have shown a marked increase in synaptic contact in this region during the first weeks of postnatal life. Therefore, the occipital lobe could be particularly prone to damage due to due to glucose availability during this period of continuous remodeling (141). A meta-analysis involving 1657 infants found that in early childhood (2-5 years), exposure to neonatal hypoglycemia was not associated with neuro developmental impairment but was associated with visual-motor impairment and executive dysfunction (109).

Major long-term effects seem to appear at a later age (145). In fact, in mid-childhood (6-11 years), neonatal hypoglycemia was associated with neuro-developmental impairment and low literacy and numeracy (145). Another study compared the long-term outcomes of preterm infants with a history of hypoglycemia with those of normoglycemic controls at 3 to 18 years of age, concluding that no significant differences were observed in cognitive or academic skills between the control and affected groups at any age (146).

## 5 Conclusions

Glucose is the most important substrate for proper brain functioning and development. Its metabolism changes over the years according to a pattern related to the acquisition of more precise ability to interact with the environment. The increased glucose metabolism demonstrated in childhood is related to the need of creating new brain structures and connections; therefore, alterations in glucose homeostasis will inevitably be associated with changes in the development of the Nervous System.

Several studies have highlighted how the alteration of glucose homeostasis leads to a reduction in gray and white matter volume and to brain metabolism alterations. Although the mechanism of brain damage caused by dysglycemic conditions is not yet completely known, structural abnormalities have been predominantly associated with hyperglycemic conditions. This is particularly interesting in relation to the therapeutic habit of preferring higher blood sugar levels in children with T1D, rather than risk of incurring unwanted hypoglycemia.

Our knowledge on the impact and outcomes of dysglycemia on the infant brain are still partial and are almost all derived from studies performed on children diagnosed with T1D, a

disease characterized also by insulin alteration and episodes of hyperketonemia. Therefore, it is possible that some of the anomalies found are due to the much more complex pathological picture.

Recent studies about the impact of dysglicemia on cognitive outcomes found that children with T1D had a worse outcome in different cognitive domains, including intelligence quotient, abnormalities in learning and memory and differences in executive functions. Moreover, since differences in brain structure and functionality were also found after a single episode of DKA, one might speculate about the importance of performing screening programs for T1D in order to protect children from the neurological consequences caused by such glycemic alterations.

Data available in newborns are discordant as well, and the study of this population is made even more difficult by the heterogenicity of the comorbidities analyzed in the different trials. However, both hypo and hyperglycemia may cause neurologic injury, which is expressed by a wide range of structural and cognitive abnormalities. Therefore, in high-risk infants it is important to promote strategies to maintain euglycemia and to continuously monitor glucose levels in order to reduce the severity and duration of episodes of dysglycemias.

Further research is necessary in order to improve the understanding of the effects, outcomes and mechanisms underlying brain changes following dysglycemia. New imaging methods, increasingly precise and less invasive, will also improve knowledge in this important field of pediatric research and clinical care.

## Author contributions

Conceptualization, MC and FC. Writing—original draft preparation, MC, EG and RT. writing—review and editing, EG; supervision FC. All authors contributed to the article and approved the submitted version.

## Conflict of interest

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

## Publisher's note



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# Early and precocious puberty during the COVID-19 pandemic

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During the year 2020, the COVID-19 pandemic rapidly became a severe health emergency worldwide. In order to contrast the spread of the novel SARS-CoV-2, many countries implemented extraordinary restrictive measures, such as a strict lockdown and school closures. The pandemic had a great impact on children and adolescents' daily life, leading to a much more sedentary lifestyle, to larger use of electronic devices and to an increase in stress-related symptoms. These conspicuous changes acted as disruptors of children's normal development. Since the beginning of the pandemic, many studies reported an increase in the number of precocious puberty cases as well as a faster progression rate of puberty itself, if compared to the pre-pandemic years. In this review, our aim was to evaluate the incidence of new cases of early and precocious puberty during the COVID-19 pandemic, analyzing variations in the timing of puberty and in pubertal progression rate, and to investigate the role of environmental and lifestyle factors during the pandemic in modulating the physiopathology of pubertal development. While a direct effect of SARS-CoV-2 infection remains, at the moment, a remote hypothesis, both physical and psychological factors related to the pandemic seem to have a role in triggering GnRH pulsatile secretion leading to earlier pubertal onset. It is indeed important to stress the need to clarify the exact role of COVID-19 in early pubertal onset comparing data from all over the world; long-term comprehensive studies are also pivotal to explain whether this phenomenon will continue while we resume pre-pandemic habits.

KEYWORDS

**precocious puberty, early puberty, puberty, secular trend, GnRH, COVID-19, BMI, lifestyle**

## 1 Introduction

During the first months of the COVID-19 pandemic, many governments implemented social distancing measures in order to contain viral diffusion; national lockdowns were the strictest measures. School closure and stay-at-home policy resulted in increased psychological stress (1), in the overuse of electronic devices and into a more

sedentary lifestyle. Increases in body weight and BMI were reported among children and adolescents (2).

Since the beginning of 2020, several pediatric endocrinology centers observed an increase in referrals for suspected precocious puberty and in the number of central precocious puberty (CPP) and rapidly progressive puberty cases, mainly in girls. Many of those children needed to start therapy with GnRH analogs.

This new trend was first reported in November 2020 in Meyer Children's University Hospital, Florence (3); many reports promptly came from other countries all over the world.

In the following paragraphs, after describing the mechanisms underlying pubertal development and the main causes of central and peripheral precocious puberty, the results of these studies will be examined to assess the influence of many aspects potentially linked with precocious puberty and rapidly progressive puberty. Both direct effects of SARS-CoV-2 infection and physical and psychological changes related to the pandemic will be investigated as potential underlying causes.

## 2 Puberty

Great advantages in understanding the physiology of puberty have been made in the last decades. Puberty can be defined as the transition period between childhood and adulthood. Development of secondary sexual characters and gonadal maturation happen during this phase, thus leading the individual to the achievement of reproductive capacity (4). Puberty is controlled by the hypothalamic-pituitary-gonadal axis.

A transient activation of the hypothalamic-pituitary-gonadal axis happens however from the first week of life and achieves its peak during the first 3-6 months of life leading to high gonadotropin and sex steroid levels. This postnatal activation of the axis, called "minipuberty", allows for sexual organs maturation in both boys and girls (5). The transient rise in estradiol and testosterone levels is generally not followed by clinically visible changes. In rare cases, it may lead to clinically visible signs such as vaginal bleeding and transient breast budding in girls or palpable testicular growth and development of pubic hair in boys. Minipuberty grants the initial development of genital organs in both males and females, assessing the basis for their future sexual maturity and fertility. However, the exact role of this process needs to be further explored and light must be thrown on the exact mechanisms that make the hypothalamic-pituitary-gonadal axis turn itself off until puberty (5, 6).

As for the reactivation of the axis, it is probably due to a dense interaction between genetic, epigenetic, and neuroendocrine factors (7). Nutritional status, body weight, exercise and exposure to endocrine disruptors play a role too (8, 9).

Pubertal development is the result of increased secretion of hypothalamic gonadotropin-releasing hormone (GnRH), gonadotrophins and, subsequently, gonadal sex steroids. Gonadotropins (luteinizing hormone, LH, and follicle-stimulating hormone, FSH) are released in a pulsatile way from the pituitary gland as a response to GnRH stimulation. Studies demonstrate that what increases at puberty is GnRH pulse amplitude rather than its frequency (10, 11). Particularly, what triggers pubertal onset by stimulating GnRH pulse and gonadotropin secretion are the hypothalamic kisspeptin neurons, located in the arcuate nucleus of the hypothalamus (12). Authors demonstrated that mutations in GPR54, a G-protein coupled receptor for kisspeptin, cause hypogonadotropic hypogonadism and alter normal gonadotropin secretion and normal pubertal physiology. Mutations at different sites of the KISS1 gene, the gene encoding for kisspeptin, lead to pubertal absence or pubertal delay too (13, 14).

After the increase in amplitude of GnRH pulse, the rise in LH and FSH levels enhances the production of sex hormones by the gonads. Sex hormones, mainly estrogens, also mediate effects on growth rate, central nervous system functioning, bone mineralization (15).

Although there can be variations in the timing and tempo of puberty in boys and girls, the underlying mechanisms are the same. To assess pubertal development, physicians use detectable changes in secondary sexual characteristics to represent the degree of pubertal maturation. These modifications, described by Marshall and Tanner (16, 17), concern the degree of physical maturation of breasts and pubic hair in girls and of genitals and pubic hair in boys. The progression of these physical characteristics is described in five maturational stages, going from prepubertal stage to the achievement of full sexual maturity.

In females puberty usually starts between age 8 and age 13. Even though puberty pattern can vary, in most cases the first event is the thelarche (the onset of secondary breast development, Tanner stage B2), followed by pubarche (the first appearance of pubic and axillary hair). In the meanwhile there is an increase in linear growth that reaches its peak in stage 4. At this point, the menarche can happen. In some studies, it is debated if pubarche without breast development may represent, in girls, the true onset signal of pubertal development (18). In males, puberty starts between age 9 and age 14. The first marker of pubertal onset is a testicular volume size ≥4 mL by the Prader orchidometer (Tanner stage G2), followed by the growth of pubic and axillary hair and by the growth of the penis. During Tanner Stage 3 males usually undergo peak height velocity (19).

## 3 Precocious puberty

Precocious puberty is traditionally defined as the appearance of secondary sex characters before 8 years of age in girls and 9 years of age in boys (20). Precocious puberty needs to be

differentiated from incomplete variants of puberty such as premature thelarche, premature adrenarche and premature menarche (21). Early puberty can be defined by the presence of secondary sexual characteristics between age 8 to 10 years in girls and 9 to 11 years in boys (22, 23) and is usually a condition that does not require therapy as it is often compensated by a longer duration of puberty itself and final adult height is normal (24, 25).

Based on the nature of its etiology, precocious puberty can be defined central or peripheral.

CPP derives from earlier activation and maturation of the hypothalamic-pituitary-gonadal axis. CPP happens through the same stages as normal pubertal development and is a disease with female preponderance. While the idiopathic etiology is the most common cause in girls, underlying tumors or injuries of the central nervous system (CNS) are more common in boys (26, 27). Recently, mutations in the kisspeptin family have been identified in cases of CPP (26). The most relevant conditions associated with the development of CPP are:

- CNS tumors;
- CNS injuries;
- Genetic variants and syndromes;
- Familial forms of CPP.

Peripheral precocious puberty (PPP) is less frequent than CPP and results from sex steroid exposure without the activation of GnRH pulsatile secretion. PPP can be acquired or congenital. Some important causes of PPP are (28–30):

- Congenital adrenal hyperplasia (CAH);
- McCune Albright syndrome;
- Familial male-limited precocious puberty (FMPP or testotoxicosis);
- Sex steroid secreting tumors;
- Exogenous exposure to sex steroids;
- Van Wyk Grumbach syndrome (hypothyroidism, precocious puberty, ovarian cysts).

In CPP, treatment with GnRH agonist is considered safe, without significant adverse effects, and can be administered based on the progression of puberty and on the age of the child to stop the development of secondary sex characteristics and to preserve final adult height. Late onset of treatment (especially in girls aged more than 6), advanced bone age, shorter duration of treatment are some of the factors linked with less success in preserving adult height (31).

In PPP the clinical presentation and the treatment of choice depend on the underlying cause. The primary purpose of therapy in PPP is to remove the extra source of sex steroids thus restoring prepubertal values of sex hormones (32).

Precocious puberty has been, in the past decades, a matter of concern as the decline in age at puberty has been associated in both boys and girls with several health risks and adverse behaviors. Changes in pubertal timing are related to rapid growth and reduced final adult height (33) and to a higher risk of developing characteristics of metabolic syndrome (34). Precocious puberty has been also linked with increased rate of reproductive tract cancers in adult life such as breast cancer (35). From a behavioral perspective, precocious puberty and rapid pubertal progression have been linked to more risky behaviors, earlier sexual activity, low self-esteem, psychosocial vulnerability (36, 37).

In the past decades a secular trend in puberty was observed and the average age at menarche decreased from 17 years in the mid-19$^{th}$ century to approximately 13 years in the mid-20$^{th}$ century (38–40). More recently the general decline in age at puberty has been accompanied by a simultaneous increase in number of referrals for precocious puberty.

A study conducted on more than 17000 girls aged 3 to 12 years in 1997, the Pediatric Research in Office Settings (PROS) study, showed a decline in the age of breast and pubic hair development. As a reply to this research, in 1999 the Lawson Wilkins Pediatric Endocrine Society proposed new guidelines to define precocious puberty (41), suggesting that girls had to be evaluated if breast or pubic hair development occurred before 7 years of age (in white girls) or 6 years of age (in African American girls). No changes were suggested for the evaluation of precocious puberty in boys as no secular trend was noticed in boys (40). Nonetheless some authors suggested a bias in the PROS study, as the population selected was not a random sample (42).

After the PROS study, European (43–45) and American (46) studies confirmed a decline in age at puberty in girls and physicians questioned the reasons behind this phenomenon. Although pubertal timing is mostly determined by genetic factors, it has been supposed that the environment has a role in causing earlier onset of puberty. There is evidence, in females, of a link between BMI, hyperinsulinemia, insulin resistance and early onset of puberty, but not always pubertal tempo (47). Endocrine disruptors can be defined as "environmental contaminants that perturb hormonal systems" (48). Recently the role of pre- and post-natal exposure to endocrine disrupting chemicals, a large proportion of which is represented by pesticides, has been discussed too as they seem to affect pubertal timing (8, 49, 50). Possible connections between increased screen time, blue light, sleep quality, melatonin levels and precocious puberty are debated too and further studies on these topics are required (51–53).

## 4 Precocious puberty during the COVID-19 pandemic

During the past two and a half years, multiple centers globally recorded an increase in CPP and rapidly progressive puberty diagnoses (Table 1).

The first reports of this trend came from Italy. A retrospective study on patients referred to the Auxo-endocrinology and Pediatric Gynecology Unit of the Meyer Children's University Hospital was published in November 2020. The Authors reported an increase in CPP cases during the first lockdown (March – July 2020); the number of new diagnoses was significantly higher if compared to the mean from the same period of time in the previous five years. They also collected data concerning the rate of pubertal progression and found that some patients who had previously received CPP diagnosis moved from a slowly progressive puberty to a rapidly progressive puberty during the first lockdown. As for the reason of this new trend, the first hypotheses concerned the increase in BMI and the augmented use of electronic devices during the first lockdown. In both CPP and rapidly progressive puberty group BMI increased significantly during the lockdown and families reported an augmented use of electronic devices (3).

A different Italian study conducted in the Endocrinology Unit of Bambino Gesù Children's Hospital in Rome compared the first 2020 months with the same months in 2019 and described a significant increase in outpatient consultations for precocious puberty. In this case, however, no significant differences in BMI were reported between the first and the second group (54).

In the outpatient clinic of pediatric endocrinology of the University of Campania Luigi Vanvitelli, CPP incidence rate was 2.5-fold higher from April 2020 to April 2021 than from 2017 to 2020. While sleep disturbance was a frequently associated comorbidity, BMI was found to be similar between the two groups. CPP cases diagnosed during the pandemic had higher levels of LH, FSH and 17 beta estradiol than CPP cases diagnosed before the pandemic (55).

A significant increase in CPP diagnoses in girls in 2020 if compared to 2019 (+16%) was also found in Trieste, Italy. No increase in BMI was found in the 2020 group. Conversely, CPP diagnoses decreased in boys (-75%): the Authors traced this trend back to the fact that clinical findings such as testicular enlargement often went unnoticed and family pediatrician visits were missed during the first lockdown (56).

Similar results promptly came from research conducted all over the world. In a study among school-aged girls in Shanghai, the incidence rate of precocious puberty in 2020 was higher than that in 2016-2019; serum concentrations of MKRN3 (makorin ring finger protein 3, a negative regulator of GnRH) were lower and GnRH concentrations were higher in girls from the 2020 group. These girls had several risk factors related to lifestyle changes that happened during the first lockdown such as weight gain (the median value of weight gain was 2 kg in 6 months), augmented screen time, reduced physical activity (57). An analogous report came from a study involving 22 medical units from 13 cities in Henan Province, China. The Authors described a three-fold increase in the number of precocious puberty cases in girls in 2020 if compared to 2019. Weight and BMI were significantly higher in the 2020 group. Other potential risk factors that arose were augmented use of electronic devices, reduced physical activity, vitamin D deficiency, consumption of processed food (58).

In a report from Turkey, the Authors highlighted how the number of CPP and rapidly progressive early puberty diagnoses increased three times in the pandemic period (April 2020 – June 2021) if compared to the pre-pandemic period (January 2019 – March 2020). Among these girls, 85% of the ones diagnosed with CPP and 67% of the ones diagnosed with rapidly progressive early puberty needed to start treatment with GnRH analogs. Interestingly, the Authors noticed that 28% of CPP and 69% of rapidly progressive early puberty cases were obese or overweight and were diagnosed during the pandemic period (59).

However the correlation with an augmented BMI was not described in other Turkish reports. In the first one, the Authors reported an increase in both CPP cases and in rapidly progressive puberty during the first months of the pandemic, with earlier age at the diagnosis and at the beginning of treatment. The role of environmental factors was considered; BMI SDS (standard deviation score), however, was similar between the two groups (60). A different study conducted among several pediatric endocrinology clinics in Turkey reported an increase in precocious puberty and significantly accelerated puberty cases during the pandemic; the need for pubertal suppression treatment with GnRH analogs was augmented too. Comparing the two groups, age at presentation and age at pubertal onset were lower in the pandemic group than those in the pre-pandemic group but there was no significant difference between the BMI SDS values of the two groups (61).

Data from a tertiary level pediatric endocrinology center in Western India are consistent with data from other countries. The Authors compared a pre-lockdown and a lockdown group of boys and girls and showed a three-fold increase in the referrals for precocity. A higher increase was reported in girls. Mean weight and BMI were not significantly different between the pre-lockdown and the lockdown group. In most cases a rapidly progressive puberty was observed with a more advanced bone age (62).

Analyzing data from the past two and a half years, some limitations arise. Most studies considered a girls only population. Male precocious puberty is less common and more often linked to organic causes. However, the role of environmental factors on male pubertal timing is still not studied and understood enough.

Many studies had a retrospective nature and did not define how many patients had a confirmed SARS-CoV-2 infection thus preventing the Authors to assess a possible direct effect of COVID-19.

While nutritional status and BMI are known factors that affect pubertal timing, not all the studies conducted during the

TABLE 1 Summary of reviewed studies about the impact of COVID-19 on puberty.

| Reference | Study type | Outcomes | Limitations |
|---|---|---|---|
| Stagi S et al. | Retrospective | -Increased incidence of newly diagnosed CPP and faster rate of pubertal progression in already diagnosed patients, during and after lockdown compared to previous years<br>-Increased BMI<br>-Augmented use of electronic devices | -Small sample size<br>-The authors did not study the increase in calorie intake nor the decrease in physical activity |
| Verzani M et al. | Retrospective | -Increase in precocious puberty cases in girls in the first six months of the COVID-19 pandemic, compared to the same period of 2019<br>-No differences in the anthropometric parameters of the two groups | -Boys were not studied<br>-Preliminary data; a potential link to specific pathogenetic factors was not studied |
| Umano GR et al. | Retrospective-prospective | -2.5-fold increase in CPP cases during lockdown if compared to the previous three years<br>-Higher rates of sleep disturbances and later bedtime in new CPP cases<br>-No differences in BMI and use of electronic devices<br>-Increased estradiol, LH, and FSH levels in girls diagnosed after lockdown | -Boys were not studied<br>-The link between sleep disturbances and CPP should be further investigated |
| Peinkhofer M et al. | Retrospective | -16% increase in CPP in girls during 2020 compared to the same period in 2019<br>-No increase in BMI SDS between early pubertal girls in 2020 if compared to 2019<br>-75% decrease in CPP in males | -Data collected from a single center |
| Chen Y et al. | Cross sectional | -Higher incidence of precocious puberty in 2020 than in 2016-2019<br>-Decreased serum concentrations of MKRN3 and ghrelin in the 2020 group<br>-Decreased physical activities, increased food consumption and rapid weight gain in the pandemic group | -Small sample<br>-Prolonged frozen storage of samples may have led to hormones degradation<br>-As a cross sectional study, the link between MKRN3 and ghrelin was not studied |
| Fu D et al. | Retrospective | -Increased incidence of precocious puberty in girls in 2020 if compared to 2018-2019;<br>-Potential link to prolonged use of electronic devices, changes in diet, decreased outdoor activities, vitamin D deficiency, increased BMI, genetic factors, endocrine disruptors. | -Small sample<br>-Boys were not studied |
| Acinikli KY et al. | Retrospective | -3-fold increase in the number of CPP and rapidly progressive early puberty cases in the first 15 months of the pandemic if compared to the same period before<br>-85% of CPP and 67% of rapidly progressive early puberty cases promptly started treatment with GnRH analogs<br>-28% of CPP and 69% of rapidly progressive early puberty cases were obese or overweight and were diagnosed during the pandemic period | -Limited follow-up period<br>-Past SARS-CoV-2 infection and many lifestyle changes potentially linked with the increased frequency of CPP were not investigated |
| Orman B et al. | Retrospective | -3-fold increase in the number of CPP and rapidly progressive puberty cases in three months of lockdown if compared to the same period from the previous year<br>-Aarly admission age in patients who were given treatment for precocious puberty<br>-Similar BMI SDS values between the two groups | -Data collected for three months only<br>-Small sample size |
| Yesiltepe Mutlu G et al. | Retrospective | -Increase in precocious puberty and accelerated puberty cases during the pandemic<br>-Increased need for pubertal suppression treatment at the time of presentation in the pandemic group<br>-Lower age at presentation and lower age at pubertal onset in the pandemic group<br>-Similar BMI SDS values between the two groups | -Lack of information on lifestyle changes |
| Mondkar SA et al. | Retrospective | -3-fold increase in the referrals for precocity during the lockdown period<br>-Significant increase in referrals in girls (3.7 times)<br>-Higher number of CPP diagnoses during the lockdown period<br>-More advanced bone age in the pandemic group | -Lack of information on lifestyle changes |

COVID-19 outbreak reported a correlation between precocious puberty and an augmented BMI. Furthermore, the potential risk factors for precocious puberty taken into account in the studies were not homogeneous.

On a side note, it has to be considered that some of the patients that received a diagnosis during the pandemic had pubertal onset prior to that time.

## 5 Direct effects of SARS-CoV-2 infection

Puberty is characterized by a broad range of physical and psychological changes necessary for the achievement of full reproductive maturity. These changes parallel modifications within the CNS (63): in a period in which the hypothalamus is so plastic, a possible direct effect of SARS-CoV-2 infection on hypothalamic cells has to be investigated.

Taking a step back, GnRH neurons in the hypothalamus share common embryonic origin with the olfactory bulb (OB): they develop outside the brain, in the olfactory placode, and then migrate to the hypothalamus during embryogenesis. Then, when this process is complete, GnRH neurons project their axons to the median eminence and from there they release GnRH (63). Some authors suggested a strong positive correlation between OB volume, pituitary length and precocious puberty (64).

Although there is no current proof of neuroinvasiveness of SARS-CoV-2 and neuroimaging during the acute infection is rare due to disease control and safety concerns, there is evidence for abnormal brain findings in autopsies and viral spread from the olfactory epithelium to the OB has been demonstrated (65). However there is currently no clue on what the dominant mechanisms in initiating puberty might be: direct OB inflammation (65), blood-brain barrier disruption (66) or cytokine storm (67).

Inflammatory cytokines may play a crucial role also stimulating N-methyl-D-aspartate (NMDA) receptors which promote GnRH pulsatile secretion through inputs from neurotransmitters such as glutamate (65, 68, 69).

## 6 Physical and psychosocial changes related to the pandemic

In the first months of the COVID-19 pandemic, when vaccines were still unavailable, containment measures such as lockdown and social distancing were effective in limiting viral transmission thus reducing case incidence and growth rate (70).

On the other hand, these measures had negative effects on both physical and psychological health in adults as well as in children and adolescents (71, 72). These new circumstances led to increased levels of stress and anxiety, greater rates of depression, and a feeling of helplessness (73, 74). Moreover, it has been noted how the COVID-19 pandemic will probably have long term adverse effects on children and adolescents' health: hence the need for longitudinal, extended research on children and adolescents during and after the pandemic (75, 76).

Psychological factors such as fear and anxiety could have played a part in the outbreak of precocious puberty cases mediating an effect on CNS mediators such as NMDA (77) and glutamate (78). The rise of anxiety levels could have triggered the activation of GABA A receptors in prepubertal subjects, inducing the activation of stress pathways that are known to be typical of pubertal onset (79). Currently available data on changes in CNS mediators during the pandemic are nevertheless not enough to account for the rise of precocious puberty cases and further studies are required.

The main lifestyle changes regarded the reduction of both indoor and outdoor physical activities during the first pandemic wave (80, 81). This more sedentary routine, together with the worsening of eating habits, intensified the burden of childhood overweight and obesity. Many studies observed an increased consumption of total packaged snacks during the day as well as of sugary food, high calory food and processed meat (82–84). As a result, not only an increase in BMI was observed (85), but there were also serious alterations in glucose and insulin metabolism, specifically in already overweight and obese children: fasting glycemia, glucose, insulin excursion were significantly higher in children during the pandemic if compared to data from pre-pandemic children (86).

Childhood obesity has been associated with the secular trend of puberty anticipation, especially in girls, observed over the last few decades (87, 88). Some authors found that the precocious onset of pubertal development in these children might be related to the presence of higher leptin levels (89), which stimulate the production of sex hormones. The rapidity of weight gain itself may conduce to higher leptin and lower ghrelin levels. In a recent study conducted among Shanghai girls, the precocious puberty group was characterized by higher serum concentrations of GnRH and lower concentrations of ghrelin, confirming the important role of ghrelin in regulating pubertal mechanisms (57).

There is also a positive correlation between hyperinsulinemia, alone or in conjunction with adiposity, insulin resistance and precocious puberty (90, 91). It has been suggested that insulin resistance, in particular, may lead to an increased bioavailability of sex hormones by reducing the levels of sex hormones binding proteins (92). The increased levels of free sex hormones may trigger pubertal onset.

Though playing an important role in the rise in precocious puberty diagnoses during the pandemic, weight gain and BMI have probably acted together with other environmental factors.

As a result of the pandemic, screen time increased in children as well as in adults. In prepubertal age, particularly, screen time increased both due to online school lessons and to the reduction of social interactions and outdoor activities.

TABLE 2 – Possible causes of increased precocious puberty rate during the COVID-19 pandemic.

| | |
|---|---|
| Direct SARS-CoV-2 infection effects | Direct olfactory bulb inflammation<br>Blood-brain barrier disruption<br>Cytokine storm |
| Physical and psychological changes related to the pandemic | Stress, fear, anxiety<br>Reduction of physical activity and time spent outdoor<br>Increased BMI<br>Alterations in glucose and insulin metabolism<br>Increased screen time and blue light exposure<br>Poor sleep quality<br>Vitamin D deficiency<br>Exposure to endocrine disrupting chemicals |

Studies reported increases in both total and leisure screen time (93), with more hours spent watching TV and using smartphones and video games.

Another condition observed during the first months of the pandemic was a decline in vitamin D levels related to the reduction of time spent outdoors and of sun exposure because of the restrictions (94). Vitamin D deficiency has been linked to diabetes, obesity and autoimmune diseases. New studies show that vitamin D deficiency can also be associated with precocious puberty, particularly in girls (95, 96). Further research on this topic is needed to better clarify this potential link.

In the past decades, sleep changes as well as melatonin levels have been linked to pubertal development and, although studies on this topic are limited, data suggest that high melatonin levels in prepubertal age have an inhibitory effect on pubertal progression (97). In another study, the levels of 6-sulphatoxymelatonin were highest in the prepubertal group of girls and lowest in girls affected by precocious puberty (53). During the pandemic, an increased rate of sleep problems (assessed by personal perception and with scientifical instruments) was reported in families (98). In children and adolescents, sleep duration, bedtime and overall sleep quality got worse during the first lockdown, and that was probably due to both psychological factors and to increased usage of digital devices (99). It is still unknown if these changes could have played a role in the rise of precocious puberty cases.

The increase in the use of disposable items could have also contributed to a greater exposure to endocrine disrupting chemicals such as bisphenol A (BPA) and phthalate esters, used as additives in the making of plastic (100, 101). Several endocrine disrupting chemicals are known to influence the onset and the progression of puberty (102).

## 7 Conclusions

During the first phase of the COVID-19 pandemic, many governments applied social distancing measures and strict lockdowns to prevent the spread of the disease. As a result, changes in children's physical and psychological health were recorded. An interesting finding was an increase not only in precocious puberty cases, but also in pubertal progression rate.

It is still not sure if SARS-CoV-2 infection can play a direct role in this event through direct OB inflammation, blood-brain barrier disruption or cytokine storm. There is instead more research on lockdown effects. Changes in lifestyle could likely have functioned as precocious puberty triggers. Changes in BMI, glucose and insulin metabolism, screen time, sleep quality, vitamin D levels, endocrine disruptors are all factors potentially linked to sex hormones modifications and early onset of puberty as well as faster pubertal progression rate (Table 2).

To further validate these hypotheses and better explain the exact role of SARS-CoV-2 infection and of the pandemic, more results from specialized centers from all over the world are expected in the near future. In the last analysis, two remaining questions arise. The first one regards parents' role, as, maybe, the increase in precocious puberty referrals could be linked to closer monitoring of children during the pandemic. Parents working from home may have spent more time with their children and this may have helped in identifying in advance pubertal changes. The second one is about the duration of this phenomenon and whether it will continue or not while we resume pre-pandemic habits.

## Author contributions

The authors confirm contribution to the paper as follows: SP and FC contributed to conception and design of the review. SP drafted the manuscript. FC edited and revised the manuscript. Both authors contributed to the article and approved the submitted version.

## Conflict of interest

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

## Publisher's note



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# Monitoring treatment in pediatric patients with 21-hydroxylase deficiency

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21-hydroxylase deficiency (21-OHD) is the most common form of congenital adrenal hyperplasia. In most developed countries, newborn screening enables diagnosis of 21-OHD in asymptomatic patients during the neonatal period. In addition, recent advances in genetic testing have facilitated diagnosing 21-OHD, particularly in patients with equivocal clinical information. On the other hand, many challenges related to treatment remain. The goals of glucocorticoid therapy for childhood 21-OHD are to maintain growth and maturation as in healthy children by compensating for cortisol deficiency and suppressing excess adrenal androgen production. It is not easy to calibrate the glucocorticoid dosage accurately for patients with 21-OHD. Auxological data, such as height, body weight, and bone age, are considered the gold standard for monitoring of 21-OHD, particularly in prepuberty. However, these data require months to a year to evaluate. Theoretically, biochemical monitoring using steroid metabolites allows a much shorter monitoring period (hours to days). However, there are many unsolved problems in the clinical setting. For example, many steroid metabolites are affected by the circadian rhythm and timing of medication. There is still a paucity of evidence for the utility of biochemical monitoring. In the present review, we have attempted to clarify the knowns and unknowns about treatment parameters in 21-OHD during childhood.

KEYWORDS

**urine pregnanetriol, 17-hydroxyprogesterone, 21-hydroxylase deficiency, congenital adrenal hyperplasia, first morning urine sample**

## Introduction

21-hydroxylase deficiency (21-OHD), the most common form of congenital adrenal hyperplasia, is an autosomal recessive disease caused by mutations in *CYP21A2* and has an incidence of 1:15,000-18,000 births (1, 2). *CYP21A2* encodes adrenal steroid 21-hydroxylase (P450c21), an enzyme which converts 17-hydroxyprogesterone (17OHP) to 11-deoxycortisol and progesterone to deoxycorticosterone, the precursors of cortisol and aldosterone,

respectively. The blockade of cortisol synthesis leads to corticotropin stimulation of the adrenal cortex; the resulting accumulation of the precursors is diverted to sex hormone biosynthesis (Figure 1).

Newborn screening, now performed in many developed countries, leads to early diagnosis of 21-OHD, especially of the classical phenotype. Moreover, recent advances in genetic testing have solved certain problems related to diagnosing 21-OHD. On the other hand, many challenges related to treatment remain. Managing patients with 21-OHD using glucocorticoids (GCs) is not optimal, and additional treatments have been proposed (3–5), such as the androgen antagonist, flutamide; the aromatase inhibitor, testolactone; the P450c17 inhibitor, abiraterone acetate (6, 7); the corticotropin-releasing factors, receptor 1 antagonist crinecerfont (8, 9) and tildacerfont (10); ACTH antagonists (11, 12); and melanocortin type 2 receptor antagonist (13, 14).

Indices for managing mineralocorticoid (MC) treatment include blood pressure and serum electrolyte and plasma renin levels (3, 4). However, the optimal dosage for MC substitution has not been critically studied. Sensitivity to MC is relatively lower during the neonatal and early infantile periods than later in life owing to immature tubular reabsorption of sodium, making MC treatment difficult. There are also unresolved challenges concerning the monitoring of MC treatment.

In the present review, we addressed the following topics, focusing on challenges related to monitoring the GC treatment for 21-OHD during childhood.

1) Goals of 21-OHD treatment in childhood
2) Gold standard of monitoring pediatric 21-OHD
3) Blood sampling to monitor 21-OHD treated with GC
4) Utility of urine sampling to monitor 21-OHD
5) Future prospects

## Goals of 21-OHD treatment in childhood

The goals of treating childhood 21-OHD are preventing adrenal crisis and virilization and maintaining growth and maturation comparable to those of healthy children (3, 4). Under-treatment causes adrenal insufficiency, such as weight loss, anorexia, gastrointestinal complaints, weakness, and fatigue. Insufficient cortisol synthesis in patients with 21-OHD also leads to an impaired negative feedback drive to the hypothalamus and pituitary or increased ACTH secretion, resulting in excess 17OHP and adrenal

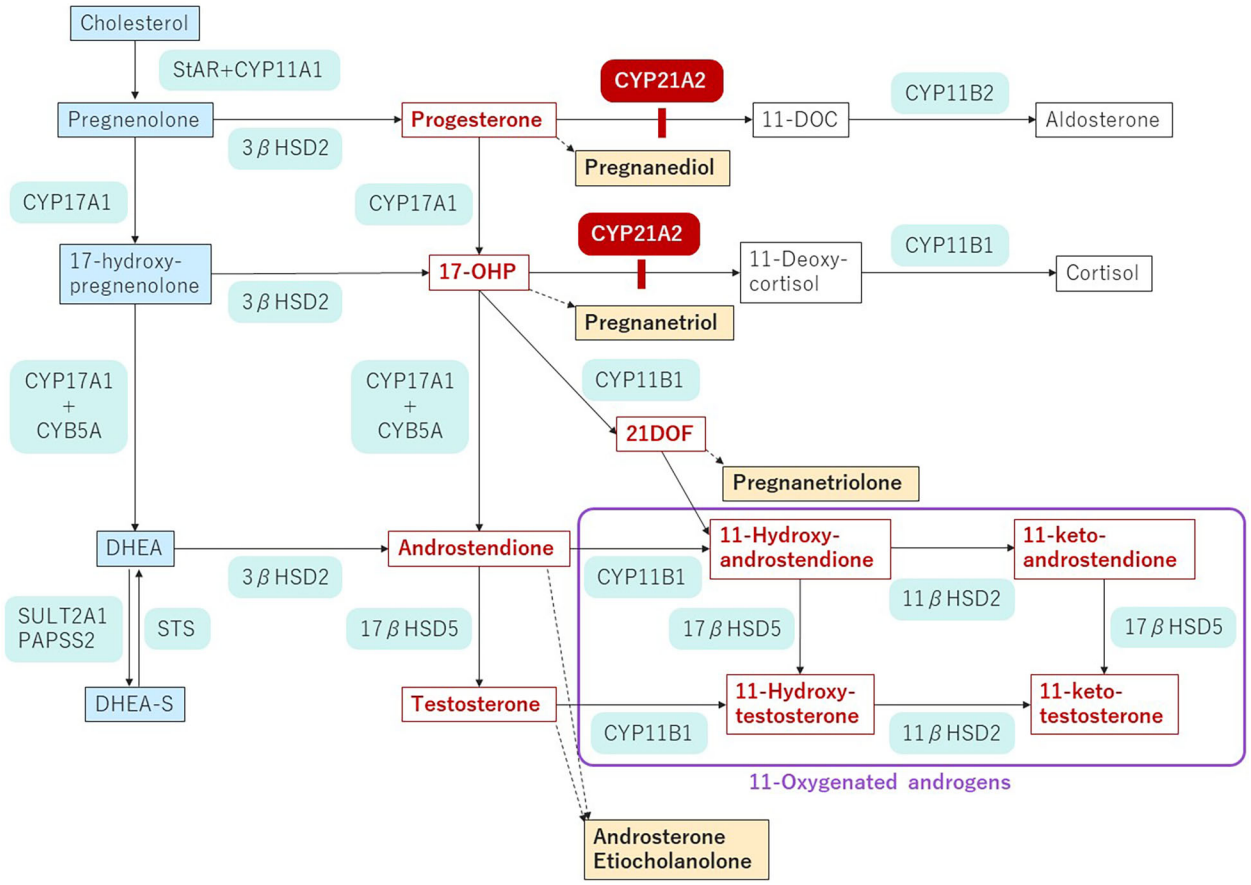


FIGURE 1
Pathway of steroidogenesis in 21-OHD.The enzyme blockade in 21-OHD is indicated by the red bar. Androgens and androgen precursors are indicated in red font. Urinary metabolites are indicated by yellow highlighting. StAR, steroidogenic acute regulatory protein; CYP, cholesterol side chain cleavage; HSD, hydroxysteroid dehydrogenase; CYB, cytochrome; DOC, deoxycorticosterone; 17-OHP, 17-hydroxyprogesterone; 21-DOF, 21-Deoxycortisol; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; STS, steroid sulfatase; SULT, sulfotransferase; PAPSS2, 3′-phospho-5′-adenylyl sulfate.

androgens. Starting in the fetal period, excess androgens induce masculinization in female patients. Abnormally increased height velocity and acceleration of bone maturation occur in both sexes. The abnormal advancement of bone maturation results in loss of growth potential and short stature. In this regard, GC therapy, which mainly consists of hydrocortisone (HDC) for children with 21-OHD, aims to compensate for the cortisol deficiency and suppress excess adrenal androgen production (3, 4). However, it is not easy to calibrate the GC dosage accurately in patients with 21-OHD. Even if physiological HDC can be supplemented to counteract the cortisol deficiency, it cannot suppress ACTH secretion completely. Therefore, HDC requirements exceed the physiological dosage whereas excessive treatment causes iatrogenic Cushing's syndrome characterized by short stature, obesity, and hypertension. Taken together, both under- and over-treatment can lead to a lower quality of life.

Patients with 21-OHD often attain final height (FH) that is significantly lower than their parentally determined, target height. In a 2001 meta-analysis of final height based in 18 studies conducted between 1977 and 1998 (15), the mean final height SD score (FH SDS) was -1.57 for male patients and -1.24 for female patients for an average of -1.37. A second meta-analysis of 35 studies conducted between 1966 and 2007 showed an average FH SDS of -1.38 and a corrected FH SDS of -1.03 (16). A recent study reported mean FH SDS values of -0.4 to -1.4 (17–23). Good compliance, early diagnosis (age <1 year), and short duration of HDC treatment in the peripubertal period reportedly improved FH (15, 19, 20, 22). For example, FH positively correlated with height at age 2 years in both sexes (24). It also positively correlated with the age at menarche in female salt-wasting (SW) and simple virilizing (SV) groups (24). Furthermore, the major determinant of FH differed among the various phenotypes of 21-OHD (24). Other critical factors determining FH are age at puberty initiation and pubertal height gain (25–29). In addition, most studies of FH revealed a sex difference; FH SDS was higher in female patients than in male patients (15, 19–22). This difference was thought to stem from the tendency toward a later diagnosis in male patients with the SV form.

Achieving normal pubertal development is another important goal for 21-OHD patients, in whom the onset of puberty varies (17, 24, 30). Puberty began significantly earlier in female patients with 21-OHD with the SW and SV forms than in those with the non-classic forms or the general female population whereas puberty in male patients began earlier only in patients with the SV form (24). In another study enrolling more subjects, patients of both sexes had earlier than normal onset of puberty (30). On the other hand, yet another study reported that breast development or menarche did not occur earlier in female patients with 21-OHD (17). These studies presumably referred to central puberty; however, it should be noted that some of the studies failed to describe the inclusion criteria for androgen excess.

Pubertal height gains also differed among previous reports. One reason for this difference is that the definition of pubertal height gain varied among studies. Nonetheless, whatever the definition, several studies reported less pubertal height gain in patients with 21-OHD than in a population of healthy subjects (17, 24–29). The latest study, in which all the subjects received a diagnosis of 21-OHD in the neonatal period, demonstrated that the pubertal growth spurt was similar to that of healthy children of both sexes (23). This apparently normal pubertal development suggests that early diagnosis *via* newborn screening enabled normalization of pubertal height gain and FH. However, no studies have as of yet compared differences in pubertal height gain and FH before and after the introduction of newborn screening.

In adolescents, irregular menstruation and testicular adrenal rest tumors (TART), which are associated with poorly controlled disease, are also of clinical concern. Female patients in whom 21-OHD is well controlled usually experience normal menarche (30). Therefore, irregular menstruation rarely persists in this condition. On the other hand, the prevalence of TART reportedly increases to 20-30% from ages 10 to 18 years (31–33). Regular testicular ultrasonogram screening for TART is recommended every year to two years in male patients aged 10 years or older (3, 4). It remains to be determined whether episodes of poor disease control can influence the development of TART and later gonadal function.

## Gold standard of monitoring pediatric 21-OHD

Calibrating medication dosages for 21-OHD is difficult (3, 4, 34–37). The appropriate dosage for maintenance therapy differs among individuals for various reasons, including differences in disease severity. Auxological data, such as height, body weight, and bone age, are considered the gold standards for monitoring 21-OHD especially during prepuberty (3, 4, 25, 38, 39). The importance of height has already been mentioned above; height can be accelerated or decelerated depending on the HDC dosage. Similarly, weight can be increased by overdosing with HDC while it may be decreased or maintained at an appropriate level with lower dosages. Children older than age 1.5 years should be monitored auxologically every four months or more frequently after any change in dosing (3, 4).

Bone age can also be used as a marker. Generally, bone age can be advanced or retarded in the course of undertreatment or overtreatment of HDC, respectively. At least two points pertinent to HDC use need to be kept in mind: first, bone age should be assessed after age 2 years because even in untreated patients with classic 21-OHD, bone age does not advance until age 1-1.5 years (40). Second, during puberty bone age alone cannot be used as a clinical marker because it cannot accurately predict the FH (23). Generally, a once yearly bone age evaluation is considered sufficient, but evaluations should be performed twice yearly if the growth rate shows a rapid change, or the patient enters puberty (4).

Besides auxological data, clinicians should be alert to the physical signs of skin and mucosal hyperpigmentation and virilization (onset of pubic hair growth, apocrine odor). In addition, signs of GnRH-dependent precocious puberty, such as breast development or testicular enlargement, should be monitored because elevated adrenal androgens may activate the hypothalamic-pituitary-gonadal axis (3).

## Blood sampling to monitor 21-OHD treated with GC

A disadvantage of using auxological data is that the data require months to a year to evaluate. Therefore, a method of monitoring for a shorter period is needed.

Serum 17OHP and androstenedione are traditional indicators of adequate GC treatment for 21-OHD (3, 4). Their values can be obtained after a much shorter monitoring period (hours to days) than is the case with auxological data. ACTH values are not helpful because of fluctuations due to the circadian rhythm (Table 1). The utility of monitoring the value of androgens, such as testosterone and DHEA-S, has not been sufficiently studied. Some recent studies have found that metabolites, such as 21-deoxycortisol and 11-oxygenated androgens, may reflect adrenal androgen precursor production (41–43). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) allows us to measure several adrenal androgen precursors simultaneously but is not yet widely available (Table 1). The latest international guidelines do not provide specific target levels for steroid measurement because laboratory reference ranges and timing of sample collection vary and the whole clinical picture must be considered (3). However, the target values provided by some studies may be of use to clinicians.

ACTH and cortisol secretions fluctuate with the circadian rhythm, with levels normally increasing in the early morning (34, 44). The timing of GC administration can also influence their values (45). Serum 17OHP also follows the rhythm and is similarly affected by medication. Therefore, it is preferable to measure serum 17OHP regularly before administering GC early in the morning (3, 4). The target range of serum 17OHP immediately before the morning GC administration is reportedly 4-12 ng/mL in both children and adults (44) and <5.9 ng/mL during puberty (18). However, these target ranges were arbitrarily determined rather than being based on auxological data (34, 44). The latest study of the topic demonstrated a 17OHP cut-off value >4.3 ng/ml with a sensitivity of 85.48% and specificity of 37.59% in patients younger than 18 years with poorly controlled disease, as defined by an accelerated growth rate (46). It is noteworthy that most 17OHP values used as a clinical marker were obtained by immunoassay. A disadvantage of monitoring using the 17OHP value is the difficulty of sampling blood in hospitals in the early morning, which is outside the regular staff working hours (Table 1). To resolve this difficulty, several studies have examined dried blood spot (DBS) samples, especially those recently used with LC-MS/MS (47–50), for their utility for monitoring purposes. However, all these studies were small-scale. Moreover, the use of DBS presents several problems. First, DBS analysis cannot simply replace serum analysis until its robustness has been documented in larger comparative studies and DBS-specific reference ranges have been established. Second, DBS sampling may be difficult to do at home for non-medically trained individuals, especially young pediatric patients (51).

Standardizing 17OHP values is crucial. Currently, two methods of measuring 17OHP exist, namely, the immunoassay and mass spectrometry. However, these methods are not without their challenges. Cross-reactivity with other steroid compounds, especially in neonates, may affect the results of an immunoassay. Further, each assay uses a different antibody, leading to variability in the results (Table 1). On the other hand, LC-MS/MS enables accurate measurement of the absolute value of 17OHP and other steroid metabolites (52–54). The optimal therapeutic target range of 4-12 ng/

TABLE 1 Pros and Cons of each biochemical measurements.

| Sample | Measurement | Pros | Cons |
|---|---|---|---|
| Serum | Common points in the items below | 1) Inexpensive<br>2) Established in clinical care<br>3) No age limit; available for infants | 1) Reflects a shorter period than a urine sample<br>2) Diurnal fluctuations due to the circadian rhythm and medication |
| | 17OHP by immunoassay (17OHP by LC-MS/MS is still at the research stage) | 1) Target range of early morning 17OHP value reported<br>2) Recommended in guidelines | 1) No studies based on auxological data<br>2) Difficulties in early morning serum sampling |
| | Androstenedione by immunoassay | 1) Recommended in guidelines | 1) Target range not based on auxological data<br>2) Not available in some countries |
| | ACTH by immunoassay | | 1) Variability due to circadian rhythm and stress caused by puncture<br>2) Not recommended in guidelines |
| DBS | 17OHP by LC-MS/MS | 1) Ease of sampling in early morning<br>2) Feasibility in remote medical care | 1) DBS-specific reference ranges have not been established<br>2) Not recommended in guidelines |
| Urine | Common points in the items listed below | 1) Non-invasive<br>2) Reflects a longer period than a serum sample; overnight in the first morning sample and daily in the 24-hour sample<br>3) Accurate and standardized | 1) Difficulty with obtaining samples from infants |
| | Pregnanetriol by GC-MS | 1) Target range based on prepubertal auxological data | 1) Requirement for time-consuming derivatization steps |
| | GC-MS steroid metabolome analysis | 1) Target range based on prepubertal auxological data | 1) Not available in some countries<br>2) Requirement of time-consuming derivatization steps |
| | LC-MS/MS steroid metabolome analysis | 1) No time-consuming derivatization steps | 1) Expensive<br>2) Not yet established in clinical care |

mL for morning serum 17OHP described above (3, 4, 34, 44) was obtained using older, radioimmune assays. It is still uncertain whether the 17OHP values obtained in this way correlate with those obtained with LC-MS/MS (14, 51). Thus, standardization of immunoassay results is needed for LC-MS/MS to become more widely accepted as a measurement method.

## Utility of urine sampling to monitor 21-OHD

In clinical practice, urine sampling is more feasible, is easily done periodically, and has the added advantage of being minimally invasive. Moreover, urine samples may better reflect longer-term conditions than blood samples (Table 1). At least three studies using urine samples investigated the optimal monitoring based on auxological data (55–57).

### Pregnanetriol

Pregnanetriol (PT) is a urinary metabolite of 17OHP. Measuring PT by gas chromatography-mass spectrometry (GC-MS) has been proposed as a form of monitoring treatment of 21-OHD (55, 56, 58). Our previous studies, based on the auxological data of height velocity, body weight, and bone age, demonstrated that the morning urine PT value can be used as an index of control in prepubertal patients with 21-OHD (55, 56). In these studies, the criteria for good disease control included maximal changes in the height SDS < ± 0.2/year during the prepubertal period (Tanner stage 1) and changes in body weight SDS < 0.5/year during late puberty. Based on the criteria, the PT level during periods of good disease control ranged from 1.2 to 2.1 mg/m$^2$/day (95% confidence interval [CI] for the mean) in 24-hour urine samples (55) and 2.2 to 3.3 mg/gCr (95%CI) and 0.59 to 6.0 mg/gCr (the 10th - 90th percentile) in the first morning urine (56). In conclusion, these ranges could be used as an index of optimal control.

In a recent, prospective study, the first morning PT levels before morning medication significantly correlated with the blood 17OHP value obtained from DBS on filter paper (59). Early morning urine collection, which provides urine data from late night to early morning, is more suitable for repetitive measurements than 24-hour urine collection (Table 1).

### Urinary steroid profile by GC-MS

Two studies reported a correlation between blood 17OHP and urinary steroid metabolites on GC-MS analysis for 24 hours (45, 58). Urinary steroid metabolites, including the PT/tetrahydrocortisone ratio, the sum of three 17-hydroxyprogesterone metabolites/the sum of three cortisol/cortisone metabolites ratio, 5α-pregnane-3α, 17α-diol-20-one (a backdoor pathway metabolite), significantly correlated with the early morning 17OHP value.

Most recently, the use of growth rate was recommended as a basis for determining the target value of urinary steroid metabolite excretion in prepubertal children with 21-OHD (57). The target range for the androgen metabolite z-score and the hydrocortisone metabolite, tetrahydrocortisol, was 0.164-0.512 and <1,480 μg/m$^2$ body surface area/day, respectively. The study also reported the utility of metabotyping in urinary steroid metabolome analysis using 24-hour urine samples (60). This metabolome analysis recognizes four, unique metabotypes, each with a specific signature, characterized by differences in the cortisol metabolite and androgen metabolite values. Thus, this method may enable the classification of patients into an appropriate, overtreated, undertreated or treatment failure group. However, measuring GC-MS is not feasible at all institutions or in all countries (Table 1).

### Urinary steroid profile by liquid chromatography-tandem mass spectrometry

GC-MS analysis is the mainstay of urinary multisteroid profiling (3). Recently, Pussard et al. developed a novel, more extensive LC-MS/MS method for measuring the urinary value of 23 steroids, which demonstrated a close correlation between morning plasma and urinary 17OHP (61). Patients with well-controlled disease had a plasma concentration of 17OHP below 20 ng/mL and a normal androstenedione level before morning medication, indicating lower levels of steroid upstream of the 21-hydroxylase defect and a lower level of androgens (androstenedione, testosterone, and the sum etiocholanolone + androsterone). It is noteworthy that the study used morning spot urine samples as in a previous study of PT (56). If established, this can be a good monitoring method because morning spot urine sampling is feasible and suitable for periodical monitoring. LC-MS/MS method is promising because it is not as time-consuming as GC-MS. Its main drawback is its limited availability (Table 1).

## Future prospects

Optimizing 21-OHD treatment in children requires establishing a monitoring method that combines auxological data with biochemical measurements. Ideally, the optimal range of each biochemical indicator would be based on auxological data. Prospective studies of biochemical monitoring based on auxological data can further validate the retrospective results reported thus far.

Little is known about treatment parameters during puberty. Although the latest guidelines do not mention the management of 21-OHD during puberty (3, 4), two, retrospective studies discussing the optimal treatment method for pubertal patients (17, 62) suggested that the pubertal, growth-suppressing effects of HDC outweigh the negative effects of elevated androgens, and that the HDC dosage during puberty should not exceed 17 mg/m$^2$/day to optimize FH. However, other studies have not focused on biochemical monitoring during puberty (23–29). It may be difficult to conduct a prospective study of this matter because of the heterogeneity in pubertal progression. Research on monitoring the disease in the neonatal period and infancy faces similar challenges.

Finally, it is unknown how the results of monitoring in the neonatal period, childhood, and adolescence indicating disease control in each period may eventually affect the disease status in adulthood, including complications and quality of life. For example, it remains to be seen how quality of life and complications in patients with 21-OHD improve in adulthood if the condition is diagnosed at an early stage, such as *via* neonatal screening, to enable early intervention. Does poor biochemical control in childhood and adolescence relate to infertility in adulthood? To this and other questions, patients with 21-OHD need to be followed-up continuously. In this sense, a registry system is essential for future research. A high-quality registry system can provide clues to better monitoring methods. Several countries have already established their own, whole case registry system and produced meaningful research outcomes (23, 63–65). If the challenges of monitoring described above can be solved, treatments can be refined to enable patients with 21-OHD to receive appropriate management over the course of their whole life.

## Author contributions

TI and YH contributed substantially to the conception and design of the study, drafting of the manuscript, and its review; have given their final approval of the version submitted; and agree to be accountable for the accuracy and integrity of its content. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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

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# Impact of 6-month triptorelin formulation on predicted adult height and basal gonadotropin levels in patients with central precocious puberty

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**Background:** Triptorelin, a long-acting gonadotropin-releasing hormone (GnRH) agonist, is available in 1-, 3-, and 6-month formulations to treat central precocious puberty (CPP). The triptorelin pamoate 22.5-mg 6-month formulation recently approved for CPP offers greater convenience to children by reducing the injection frequency. However, worldwide research on using the 6-month formulation to treat CPP is scarce. This study aimed to determine the impact of the 6-month formulation on predicted adult height (PAH), changes in gonadotropin levels, and related variables.

**Methods:** We included 42 patients (33 girls and nine boys) with idiopathic CPP treated with a 6-month triptorelin (6-mo TP) formulation for over 12 months. Auxological parameters, including chronological age, bone age, height (cm and standard deviation score [SDS]), weight (kg and SDS), target height (TH), and Tanner stage, were evaluated at baseline, and after 6, 12, and 18 months of treatment. Hormonal parameters, including serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol for girls or testosterone for boys, were analyzed concurrently.

**Results:** The mean age at treatment initiation was 8.6 ± 0.83 (8.3 ± 0.62 for girls, 9.6 ± 0.68 for boys). The peak LH level following intravenous GnRH stimulation at diagnosis was 15.47 ± 9.94 IU/L. No progression of the modified Tanner stage was observed during treatment. Compared to baseline, LH, FSH, estradiol, and testosterone were significantly reduced. In particular, the basal LH levels were well suppressed to less than l.0 IU/L, and the LH/FSH ratio was less than 0.66. The bone age/chronological age ratio remained stable with a decreasing trend (1.15 at the start of treatment, 1.13 at 12 months, 1.11 at 18 months). PAH SDS increased during treatment (0.77 ± 0.79 at

baseline, 0.87 ± 0.84 at the start of treatment, 1.01 ± 0.93 at six months, and 0.91 ± 0.79 at 12 months). No adverse effects were observed during treatment.

**Conclusion:** The 6-mo TP suppressed the pituitary-gonadal axis stably and improved the PAH during treatment. Considering its convenience and effectiveness, a significant shift to long-acting formulations can be expected.

KEYWORDS

central precocious puberty, gonadotrophin-releasing hormone analogue, gonadotropins, triptorelin pamoate, 6-month formulation

# 1 Introduction

Central precocious puberty (CPP) is defined as the onset of pubertal development—breast development before the age of eight in girls, or testicular development to over 4 mL before the age of nine in boys, due to premature activation of the hypothalamic-pituitary-gonadal axis (1). It occurs 5- to 10-fold more frequently in girls than in boys, and is usually sporadic. The cause is idiopathic in approximately 90% of the girls, while a structural central nervous system abnormality may occur in 26–75% of the boys with CPP (2, 3). Early pubertal changes lead to accelerated growth, bone maturation, and tall stature during childhood, often resulting in reduced adult height due to premature growth plate fusion (4). Moreover, the high sex hormone levels in girls with CPP cause early menarche, which might lead to psychosocial problems (5). However, when the loss of predicted adult height (PAH) or social problems due to early sexual development are unlikely to be significant, the need for treatment should be evaluated through periodic observation every 3–6 months (6–8).

Since the mid-1980s, long-acting gonadotropin-releasing hormone (GnRH) agonists have been the gold standard treatment for CPP. GnRH agonists stimulate the pituitary gonadotrophs continuously, leading to desensitization and decreased release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (9). Depot or slow-release GnRH agonist formulations have been developed, significantly prolonging the drug action and improving patient compliance. The demand for these drugs has been increasing, especially during the COVID-19 pandemic (10). Triptorelin, one of these drugs, is available in 1-month (3.75 mg), 3-month (11.25 mg), or 6-month (22.5 mg) formulations (1, 11, 12).

The prevalence of CPP has been increasing worldwide (13), particularly in Korea (14, 15). Six-month triptorelin pamoate (6-mo TP) was approved for CPP treatment in Korea in March 2020. However, since only a few large hospitals can prescribe it, the available data about its efficacy and safety are scarce (12, 16).

This study aimed to determine the impact of the 6-mo TP formulation on the PAH, changes in gonadotropin levels, and related variables.

**Abbreviations:** CPP, central precocious puberty; PAH, predicted adult height; 6-mo TP, 6-month triptorelin pamoate; TH, target height; SDS, standard deviation score.

# 2 Materials and methods

## 2.1 Study design and patients

The data of all patients diagnosed with CPP based on the International Classification of Diseases 10th Revision (ICD-10; code E22.8) between January 2019 and March 2022 at a tertiary university hospital in Seoul, Republic of Korea, were retrospectively reviewed. Only those who met all the inclusion criteria were included in this study.

The inclusion criteria were based on a consensus statement (1): (1) objective breast enlargement before the age of eight in girls or testicular volume greater than 4 mL before the age of nine in boys; (2) bone age over a year ahead of the chronological age; (3) pubertal LH response to a GnRH stimulation test; (4) intramuscularly administrated 6-mo TP 31 mg (22.5 mg as triptorelin; Diphereline SR 22.5 mg Inj., Ipsen, France) for at least two doses. Patients treated with the 6-mo TP formulation for less than a year or who had other organic causes for early pubertal development (obstetrical problem, brain tumor, congenital adrenal hyperplasia, hypothyroidism, or a history of cranial irradiation) or were receiving recombinant growth hormone therapy were excluded. Girls with ovarian disease diagnosed by transabdominal ultrasonography and boys diagnosed with organic brain problems by sellar magnetic resonance imaging were excluded (16).

GnRH stimulation tests were performed at diagnosis by intravenous (IV) administration of 100 μg fixed-dose Gonadorelin (Relefact; Sanofi-Aventis, Frankfurt am Main, Germany). Serum LH and FSH levels were assessed before and 30, 45, 60, and 90 minutes after stimulation using an immunoradiometric assay (BioSource SA, Nivelles, Belgium). A peak LH level above 5 mIU/mL was considered diagnostic for CPP (17, 18).

The Institutional Review Board of the Kangbuk Samsung Hospital approved this study (IRB number KBSMC 2022-12-050) and waived the requirement for informed consent owing to the retrospective design of the study. Besides, the patients' information was anonymized and de-identified before analysis.

## 2.2 Assessments

The primary outcome measure was basal LH suppression to prepubertal level. Secondary outcome measures were suppression of sex hormones, slowing the progression in the development of secondary sexual characteristics and bone maturation and increasing PAH (19).

Blood samples for random serum LH, FSH, and estradiol for girls or testosterone for boys were collected pre-injection every six months during treatment to assess gonadotropin suppression. LH and FSH levels without stimulation at diagnosis and each visit were referred to as basal LH and FSH. Effective LH suppression to prepubertal levels was defined as basal LH <1 mIU/mL (17, 20–22). Prepubertal basal FSH was defined as <2.5 mIU/mL (23), and basal LH/FSH ratio as <0.66 (24, 25). The lower limits of LH and FSH detection were 0.2 mIU/mL and 0.7 mIU/mL, respectively. Estradiol and testosterone reference values vary with the assessment method and assay used (1, 3, 5). Based on the laboratory cutoff values used in this study, estradiol above 12.3 pg/mL and testosterone above 0.481 ng/mL were considered pubertal levels (26).

At the time of CPP diagnosis, and every six months from 6-mo TP administration, the patients visited the outpatient clinic to measure height and weight, and the body mass index (BMI) was calculated. Standard deviation scores (SDS) of height, weight, and BMI were calculated according to the 2017 Korean National Growth Charts for children and adolescents (27). Bone age was measured by an experienced pediatric endocrinologist and radiologist using left wrist radiographs and following the Greulich-Pyle Method (28). The bone age/chronological age ratio was used as an index of bone age advancement (3). Sexual development was assessed using Tanner staging at each outpatient visit. Testicular volume was measured by an experienced pediatric endocrinologist using an orchidometer. Breast examination was performed through breast inspection and palpation according to the modified Tanner method (3). Midparenal height was set as the patient's target height (TH) and calculated as follows: (father's height + mother's height)/2 ± 6.5 cm (–6.5 cm for girls and +6.5 cm for boys) (29). PAH was calculated using the Bayley-Pinneau average age method (30). Adverse events were monitored during the treatment period (31).

## 2.3 Statistical analysis

Data were analyzed using IBM SPSS Statistics, Version 24.0 (IBM Corp., Armonk, NY, USA). Averages over time of hormonal parameters, bone age/chronological age ratio, and PAH SDS were compared by a linear mixed model, and an overall $p$-value < 0.05 compared at the time of diagnosis was considered statistically significant. *Post-hoc* analysis and Bonferroni corrected $p$ values were used to compare parameters between time points and at diagnosis.

# 3 Results

## 3.1 Patient characteristics

This study included 42 patients (33 females and nine males) with idiopathic CPP. The clinical, demographic, and laboratory baseline characteristics of the patients are shown in Table 1. The age at GnRH agonist treatment initiation was 8.33 ± 0.62 for girls and 9.65 ± 0.68 for boys. The starting dose was administrated as a 1- or 3-month depot TP and changed to 6-mo TP at a mean age of 8.80 ± 0.70 for girls and 9.88 ± 0.63 for boys. Changes in the formulation were made to reduce the frequency of pain resulting from the injection, increase convenience, and improve compliance. The 6-mo formulation change occurred at an average of 4 months after treatment initiation after three injections of the 1-month dosage form or one injection of the 3-month dosage form. The 6-mo TP was prescribed and administered four times in 16 patients, three times in 14, and twice in 12, with an average of 3.10 ± 0.80 times. The peak LH level following IV GnRH stimulation was 15.47 ± 9.94 IU/L at diagnosis of CPP.

TABLE 1 Clinical, demographic, and laboratory characteristics of the patients at baseline.

| Variable | Total ($n$ = 42) | Girls ($n$ = 33) | Boys ($n$ = 9) |
|---|---|---|---|
| Age at diagnosis (years) | 8.61 ± 0.83 | 8.33 ± 0.62 | 9.65 ± 0.68 |
| Age at start of 6-month formulation (years) | 9.02 ± 0.81 | 8.80 ± 0.70 | 9.88 ± 0.63 |
| BA at diagnosis (years) | 9.54 ± 1.11 | 9.27 ± 11.19 | 10.54 ± 14.05 |
| BA/CA ratio at diagnosis | 1.11 ± 0.09 | 1.11 ± 0.1 | 1.09 ± 0.08 |
| Weight (kg) | 32.41 ± 8.48 | 29.12 ± 4.04 | 44.47 ± 9.75 |
| Height (cm) | 135.36 ± 7.12 | 132.73 ± 4.74 | 145 ± 6.09 |
| BMI (kg/m$^2$) | 17.45 ± 2.89 | 16.48 ± 1.54 | 21.03 ± 3.86 |
| Weight (SDS) | 0.47 ± 1.18 | 0.29 ± 0.85 | 1.13 ± 1.89 |
| Height (SDS) | 0.80 ± 0.90 | 0.66 ± 0.85 | 1.31 ± 0.96 |
| BMI (SDS) | 0.54 ± 1.02 | 0.28 ± 0.69 | 1.47 ± 1.46 |
| TH (cm) | 164.74 ± 6.11 | 162.21 ± 3.57 | 174 ± 4.19 |
| TH (SDS) | 0.24 ± 0.7 | 0.28 ± 0.69 | 0.11 ± 0.74 |
| Tanner stage † | 2.26 ± 0.59 | 2.27 ± 0.52 | 2.22 ± 0.83 |
| Peak LH at diagnosis (mIU/L) | 15.47 ± 9.94 | 14.15 ± 9.43 | 20.30 ± 10.84 |

All values are presented as means ± standard deviation. BA, bone age; CA, chronological age; BMI, body mass index; TH, target height; SDS, standard deviation score.
†breast development scale for girls, external genitalia scale for boys using testicular volume.

## 3.2 Changes in the hormonal parameters

The basal LH level at diagnosis, 0.72 ± 0.86 mIU/mL, declined to 0.58 ± 0.69 mIU/mL by the time of formulation change. Its levels 6, 12, and 18 months after the formulation change were 0.43 ± 0.16, 0.43 ± 0.16, and 0.46 ± 0.12 mIU/mL, respectively. Basal LH level at follow-ups were lower than that at the time of diagnosis. However, this difference was not significant (overall $p$ =0.109)

The basal FSH level at the start of the 6-mo TP treatment was 2.52 ± 1.76 mIU/mL, and it was 2.06 ± 3.58, 1.74 ± 1.00, and 2. 15 ± 0.82 mIU/mL 6, 12, and 18 months later (overall $p$ = 0.049). The LH/FSH ratio remained well below 0.4 throughout treatment without any significant change (overall $p$ = 0.098). The sex hormones significantly decreased in both girls and boys (Figures 1A, B). In girls, estradiol decreased significantly from the level at the formulation change time (7.35 ± 8.28 pg/mL) to 4.00 ± 0.00, 4.48 ± 2.38, and 4.00 ± 0.00 pg/mL 6, 12, and 18 months later (overall $p$ = 0.006). In boys, the testosterone level was 0.83 ± 1.05 ng/mL at CPP diagnosis, 0.57 ± 0.85 ng/mL at formulation change, and 0.06 ± 0.03 ng/mL six months later (overall $p$ = 0.04).

## 3.3 Changes in the clinical parameters

During the treatment period, Tanner pubertal staging of our subjects remained similar to their stage at diagnosis. The mean Tanner stage before treatment was 2.26 ± 0.59 (2.27 ± 0.52 for girls, 2.22 ± 0.83 for boys). The baseline breast development before treatment was at Tanner stage 2 in 75.8% of the patients, stage 3 in 21.2%, and stage 4 in 3.00%. The breast or pubic hair Tanner stage did not change during treatment in any of the girls, and no vaginal bleeding was observed. In boys, the average testicular volume was well maintained below 4 cc through treatment.

The bone age/chronological age ratio was 1.11 ± 0.09 at diagnosis and 1.15 ± 0.09 at the start of the 6-mo TP treatment. During the 18-month 6-mo TP treatment, the bone age/chronological age ratio decreased to 1.11 ± 0.08 (overall $p$ = 0.007; Figure 2).

Mean PAH SDS for the entire cohort was 0.77 ± 0.79 at diagnosis, 0.87 ± 0.84 before the 6-mo TP treatment initiation, and 1.01 ± 0.93 six months later. Mean PAH SDS remained above the initial SDS values throughout the treatment period (overall $p$ = 0.038; Figure 3A). In girls, the mean PAH SDS was 0.61 ± 0.68 at diagnosis, 0.70 ± 0.76 at the formulation change, and 0.89 ± 0.93 six months later. Their PAH values at 6, 12, and 18 months of treatment with 6-mo TP were all higher than at diagnosis (overall $p$ = 0.004; Figure 3B). In boys, although non-significant probably owing to the small sample number, the mean PAH SDS values after 6, 12, and 18 months of treatment with 6-mo TP were numerically higher than at the time of diagnosis (1.49 ± 0.81, 1.42 ± 0.86, and 1.34 ± 0.00, respectively; overall $p$ = 0.387; Figure 3C).

## 3.4 Adverse events

Temporary induration and pain at the injection site were noted during the treatment period, but no long-lasting local

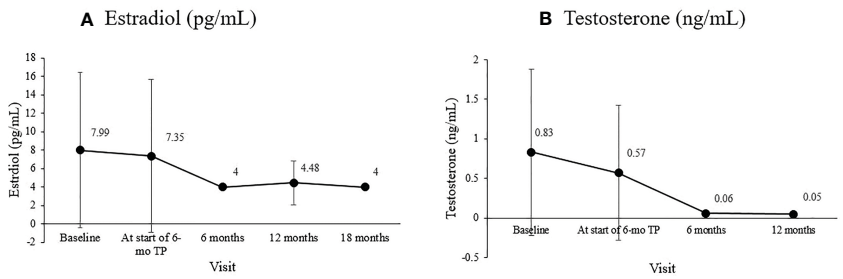


FIGURE 1
Changes in the mean sex hormone levels during treatment. **(A)** Estradiol (pg/mL; overall P =0.006). **(B)** Testosterone (ng/mL; overall p = 0.040)

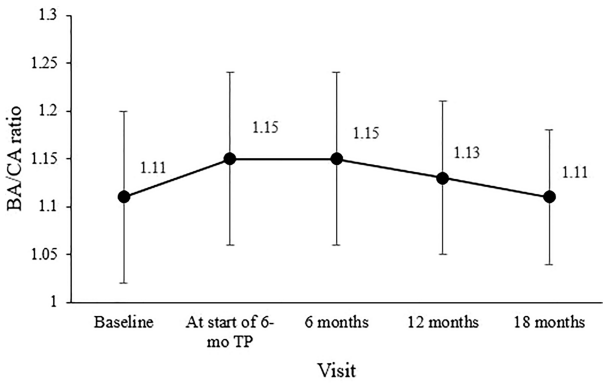


FIGURE 2
Changes in the mean bone age/chronological age (BA/CA) ratio during treatment. The overall significance was p = 0.007.

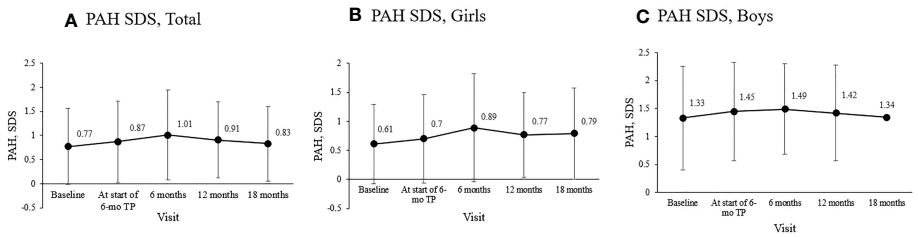


FIGURE 3
Changes in the mean PAH SDS during treatment. **(A)** The entire cohort. **(B)** Girls. **(C)** Boys. Predicted adult height, PAH; standard deviation score, SDS; TP, triptorelin pamoate

complications occurred. Other complications, such as headache, rash, gastrointestinal symptoms, and menopausal symptoms, did not occur in any of the patients during the study period.

# 4 Discussion

This study analyzed the treatment effects of a 6-mo TP (22.5 mg triptorelin) formulation in children with CPP in various aspects, focusing on changes in hormones, bone age, and PAH. The 6-mo TP formulation recently approved for CPP offers greater convenience to children than other formulations, by reducing the injection frequency; however, related research is very limited (12). The 1-month dose presentation is still the most used, and usage of the 3-month dosage form is increasing (32–34); however, there is a lack of clinical experience worldwide regarding the use of the 6-month formulation (35). It has not yet been universalized, and doubts about its efficacy and safety persist among guardians and pediatric endocrinologists (1, 35). Therefore, the 6-mo TP formulation was not used from treatment initiation in any of the cases in this study. The change to 6-mo TP was mainly made in patients previously treated with the 1-month or 3-month formulation. The shift to the 6-month dosage form in this study was made at an average of four months from starting the GnRH agonist treatment.

To our knowledge, this was the second study, following the study by Klein et al. (35) from 2016, to investigate the efficacy and safety of 6-mo TP in children with CPP. Unlike this previous study (35), ours was a long-term study (>18 months), and PAH changes during treatment were analyzed. The previous study also used a simple leuprolide stimulation test with a single 30-min post-stimulation LH sample, which has been supported in several studies (36, 37). In contrast, we used the 90-min multi-sample gonadorelin stimulation test recommended as the reference standard test for CPP in other studies (38, 39). Of course, a global consensus on GnRH stimulation test and cutoff level for the diagnosis of CPP has not yet been established.

The primary outcome measure, the basal LH has been shown by many to be suitable for monitoring the effects of GnRH agonist treatment, and basal LH <0.6 IU/L was suggested as a cutoff for adequate suppression in CPP (22, 40). Throughout treatment, the basal LH level was well suppressed to below this cutoff value, suggesting that gonadotropic axis suppression was effectively achieved (Figure 4A) (20–22, 41), the basal FSH level was maintained at a prepubertal level (<2.5 IU/L; Figure 4B) (23), and the LH/FSH ratio was maintained below the 0.66 cutoff for nonprogressive precocious puberty (Figure 4C) (24, 25, 42).

Changes in serum estradiol in girls and testosterone in boys from baseline to our study's end were consistent with gonadal suppression to prepubertal levels. The sex hormones cutoff values to differentiate prepubertal from pubertal state vary among laboratories. This study considered estradiol above 12.3 pg/mL and testosterone above 0.481 ng/mL as pubertal levels based on laboratory cutoffs and previous findings (5, 23). The baseline estradiol level at diagnosis might be at the prepubertal level in 40% of girls with CPP, as seen in our study. However, a significant decrease in basal estradiol during treatment indicates satisfactory hormonal control (1, 3, 43). Estradiol decreased from 7.35 pg/mL when we started using the 6-month formulation to an average of 4 pg/mL during the 18 months of treatment, well below the prepubertal level (Figure 1A). CPP can probably be ruled out if the baseline testosterone in boys is at the prepubertal level (26). The mean basal testosterone in our study exceeded the pubertal level but

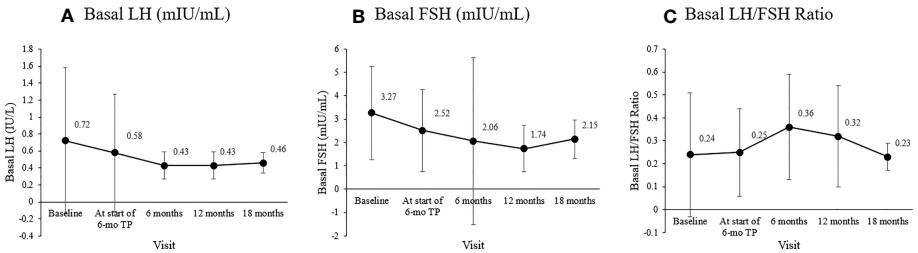


FIGURE 4
Changes in the mean basal gonadotropin levels during treatment. **(A)** Basal luteinizing hormone (LH; mIU/mL; overall p = 0.109). **(B)** Basal follicle-stimulating hormone (FSH; mIU/mL; overall p = 0.049). **(C)** Basal LH/FSH ratio (overall p = 0.098)

decreased significantly to prepubertal levels after the 6-mo TP was administered, and remained so for over a year (Figure 1B).

Secondary outcome measures were suppression of sex hormones, slowing the progression in the development of secondary characteristics and bone age maturation and increasing PAH. Clinical inhibition of sexual development during treatment was evaluated by periodic assessment of the Tanner stage. Suppression of bone age progression was determined based on a significant decrease in the bone age/chronological age ratio (3), which was maintained in this study at an average of 1.1 throughout treatment, similar to the pretreatment level. Furthermore, the bone age/chronological age ratio decreased significantly from 1.15 ± 0.09 at the 6-mo TP treatment initiation to 1.11 ± 0.07 18 months later, implying a gap between bone age and chronological age had been decreased due to suppression of bone age during treatment.

Another important end-point considered was the PAH (44), calculated based on the height and bone age measured every six months after diagnosis. PAH and PAH SDS analysis indicated that PAH SDS increased along the treatment course. This was particularly noticeable in girls. This finding suggested that the final adult height will benefit from suppressing premature growth plate fusion by the 6-mo TP treatment.

A limitation of this study was that it was not a large-scale, long-term randomized comparative trial. The 6-mo TP was considered for patients meeting the inclusion criteria who were not short, overweight, or obese. Among these, the change to 6-mo TP was considered when patients had difficulties visiting the hospital because of the COVID-19 pandemic, resided in provincial regions or overseas, had severe injection phobia, or had an allergy to the 1- or 3-month injection form that required taking antihistamines. However, most of these were excluded from the study because they received 6-mo TP only once. Some could not afford the 6-month formulation because of their private medical insurance issue. Therefore, the number of patients fulfilling the inclusion criteria was limited. Future randomized comparative studies will need to overcome these limitations and compare treatment progress with the 1- or 3-month formulation to that of using the 6-month formulation starting at diagnosis. It is also worth noting that our patients' PAH was rather high. All of our patients met the national reimbursement criteria for CPP treatment and had PAH less than TH at diagnosis. However, there is a possibility that there may have been cases in which the decision to start treatment should have been made with more follow-up. In addition, since the Bayley–Pinneau method for measuring PAH was developed in 1952 for Western children, there are parts that do not fit realistically, and adult height may have been overestimated (45).

Despite these limitations, this study analyzed various aspects of the long-term results of 6-mo TP administration in patients with CPP, including clinical, hormonal, and PAH changes. In addition, the study consistency was high because the same specialist performed its uniform treatment and examination at a single institution. Many follow-up studies are expected based on the positive effects of the 6-mo TP injection seen in this study. It is also expected that the CPP treatment paradigm will shift to the long-acting formulation.

## 5 Conclusion

This study confirmed the hypothalamic-pituitary-gonadal axis inhibitory effect of 6-mo TP. Furthermore, we confirmed that sexual development did not advance, bone age progression slowed, and the PAH SDS increased during treatment. This study provides extensive and practical information for pediatric endocrinologists by sharing our long-term clinical experience with 6-mo TP, which has not yet been widely studied.

## Data availability statement

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

## Ethics statement

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

## Author contributions

EY collected the data and drafted the manuscript. SK performed the statistical analysis and data interpretation. HJ, JYS, JWS, and DK collected and cleared the data. JK and EK contributed to the study design and takes responsibility for the integrity of the data and the accuracy of the data analysis. AY conceived and supervised the study, interpreted the results, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

## Conflict of interest

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

## Publisher's note

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# Testicular dysfunction at diagnosis in children and teenagers with haematopoietic malignancies improves after initial chemotherapy

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**Introduction:** Hematopoietic malignancies are the most frequent type of cancer in childhood. Recent advances in cancer treatment have significantly improved survival until adulthood. There is an extensive literature on the effects of cancer treatment on the gonadal axis in adult survivors of childhood cancer mainly focused on sperm production, but scarce information exists on the immediate impact of cancer and its treatment in boys.

**Objectives:** In this work, we determined the status of the hypothalamic-pituitary-testicular (HPT) axis function at diagnosis and the immediate impact of chemotherapy at the start of treatment in children and adolescents with hematopoietic malignancies.

**Subjects and methods:** In a prospective study of 94 boys and adolescents with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) or non-Hodgkin lymphoma (NHL), we determined serum AMH, inhibin B and FSH to assess the gonadotrophin-Sertoli cell component of the HPT axis, and testosterone and LH to evaluate the gonadotrophin-Leydig cell component, at diagnosis and after 3 months of chemotherapy. Secondarily, the general health state was evaluated.

**Results:** In prepubertal boys, at diagnosis, AMH, inhibin B and FSH were lower compared to the reference population, reflecting an FSH-Sertoli cell axis dysfunction. After 3 months of chemotherapy, all hormone concentrations increased. At pubertal age, at diagnosis, AMH and inhibin B were lower compared to the reference population for Tanner stage, with inappropriately normal FSH, suggesting a primary Sertoli cell dysfunction with insufficient gonadotrophin compensation. The LH-Leydig cell axis was mildly disrupted.

After 3 months of chemotherapy, inhibin B and AMH were unchanged while median FSH levels rose to values that exceeded the reference range, indicating a significant impairment of Sertoli cell function. Testosterone normalized concomitantly with an abnormal LH elevation reflecting a compensated Leydig cell impairment. General health biomarkers were impaired at diagnosis and improved after 3 months.

**Conclusion:** The HPT axis function is impaired in boys with hematopoietic malignancies before the initiation of chemotherapy. There is a primary testicular dysfunction and a concomitant functional central hypogonadism that could be due to an impaired overall health. The HPT axis function improves during the initial 3 months of chemotherapy concomitantly with the general health state. However, in pubertal boys the dysfunction persists as shown by elevated gonadotropin levels after 3 months.

KEYWORDS

AMH, inhibin B, chemotherapy, gonadotropins, hypogonadism, leukemia, lymphoma, testosterone

# Introduction

Haematopoietic malignancies represent the commonest type of cancer in childhood (1): approximately 30 cases are diagnosed per million children and adolescents worldwide every year (2). In children younger than 14 years, leukaemia's account for one third of all cancer types, with acute lymphoblastic leukaemia (ALL) as the most prevalent (3). In adolescents, lymphomas are the most frequent tumour type, representing about 23% of all cancers (3). Presently, more than 80% of haematopoietic malignancies in children and adolescents are timely diagnosed and receive effective treatment. The advances in cancer management have resulted in a significant increase in survival until adulthood, which has raised concern on fertility issues due to gonadal dysfunction (4).

While the assessment of the hypothalamic-pituitary-testicular (HPT) function is usually based upon the study of pituitary gonadotropins, testosterone secreted by Leydig cells and spermatogenesis in adults (5), in childhood mature Leydig cells are absent owing to the lack of LH stimulation and there is no sperm production (6, 7). Before pubertal onset, Sertoli cells are the most active component and anti-Müllerian hormone (AMH) and inhibin B are the preferred biomarkers to assess testicular function (5, 8–10). Serum AMH is high during infancy and childhood and declines at puberty (9, 11, 12) due to a downregulation induced by the increasing concentration of intratesticular testosterone (13). Inhibin B is high during the first 2-3 years of life, decreases though remaining clearly detectable during childhood and increases again during puberty, reflecting both Sertoli cell activity and the development of normal spermatogenesis (8, 9, 14). Both AMH and inhibin B levels are upregulated by FSH (15, 16), and inhibin B is the main responsible for the negative feedback on pituitary FSH production (17).

Most of the evidence of the effects of cancer treatment on the HPT axis relies on studies in adult survivors of childhood cancer and focuses mainly on sperm production (18); scarce information exists on the immediate impact of cancer and its treatment in boys (7, 19, 20). Research has recently addressed the importance of preventing gonadal damage and fertility preservation (21, 22). Sperm cryopreservation is the procedure of choice in adults, but this option is obviously non-existent for prepubertal boys. For these patients, testicular tissue freezing is considered a potential alternative although it is still at an experimental stage (7, 22). In any case, supporting Sertoli cells and Leydig cell precursors would need to be unscathed at the moment of tissue preservation to ensure the success of future auto-transplantation leading to sperm production and androgen secretion, essential during puberty for a normal growth spurt and bone mass accrual (4).

In this work, we aimed to determine the status of the HPT function at diagnosis in children and adolescents with haematopoietic malignancies and the immediate impact of chemotherapy at the start of treatment. In a prospective study of a large cohort of boys and adolescents, we longitudinally assessed serum levels of gonadotrophins and testicular hormones at diagnosis and after the induction phase of chemotherapy.

# Subjects and methods

## Study design and setting

We performed a prospective, analytical, study of a cohort of boys and adolescents diagnosed with haematopoietic malignancies at Ricardo Gutiérrez Children's Hospitals, a tertiary paediatric public hospital in Buenos Aires, Argentina. Patients were recruited at diagnosis for long-term follow-up in the Unit of

Haematology and referred to the Divisions of Endocrinology of Ricardo Gutiérrez, between March 2013 and February 2019. In this interim analysis, the status of the HPT axis was assessed at diagnosis and approximately after the first 3 months of chemotherapy, which corresponds to post-induction phase in the treatment of patients with ALL, acute myeloid leukemia (AML) and most non-Hodgkin lymphoma (NHL).

## Patients

### Inclusion criteria

Males aged 1-18 years with the diagnoses of ALL, AML or NHL were eligible. The diagnoses were made according to the 3rd and 4th editions of the World Health Organization Classification of Tumors of Hematopoietic and Lymphoid Tissues (23).

### Exclusion criteria

Patients were excluded if the malignancy overtly involved the central nervous system or the testes, or in any other condition known to affect the hypothalamic-pituitary-testicular axis (12, 24), in order to avoid potential confusion due to known causes of central or primary hypogonadism. Patients for whom serum samples were not available at diagnosis and after 3 months of treatment were excluded.

### Exposure and follow-up

For patients with ALL, risk stratification and treatment were assigned according to the Acute Lymphoblastic Leukaemia Intercontinental Berlin-Frankfurt-Münster 2009 (ALL IC-BFM 2009 intercontinental trial) (25). Briefly, three risk groups were defined: standard (SR), intermediate (IR) and high risk (HR), as described in Supplementary Figure 1. Protocol 8-AML-07 GATLA https://www.gatla.com.ar/images/Protocolos/LMAP_18/LMA_07/1-LMAP07.pdf was followed for patients with AML and protocol Linfoma no Hodgkin Pediátrico GATLA 2017 https://www.gatla.com.ar/images/Protocolos/LNH_2017/GUIA_DE_TRATAMIENTO_LNH_2017.pdf for patients with NHL. The cumulative doses of chemotherapy agents in the first 3 months of treatment are described in Supplementary Table 1.

## Outcome measures and definitions

At diagnosis and after 3 months of chemotherapy, the patients were evaluated by a pediatric endocrinologist and were subjected to hormonal assessments. Blood samples were obtained during hospitalization between 07.00 and 10.00 AM, immediately after diagnosis. Both at diagnosis and after 3 months, most patients received oral feeding, and some received iv fluids to maintain a euvolemic state. Patients were grouped according to pubertal stages as defined by Marshall and Tanner (26). Testicular volume was measured by comparison with Prader's orchidometer.

The main outcome measures of the study were the serum concentrations of AMH, inhibin B and FSH to assess the gonadotrophin-Sertoli cell component of the HPT axis. Secondarily we evaluated serum testosterone and LH, to assess the gonadotrophin-Leydig cell component of the HPT axis, in pubertal patients. Serum hormone levels were expressed as absolute values and as standard deviation scores (SDS) based on age- and Tanner genital stage-matched reference ranges, from a sample of 421 apparently normal males using similar hormone assays, previously published by our group (12, 27) The general health status was assessed by determining hemoglobin, C-reactive protein (CRP), and albumin serum values (28, 29). Additionally, the body mass index (BMI) was calculated as weight (kg)/height $(m)^2$, and expressed as standard deviation score (SDS) in boys > 2 years old. (https://zscore.research.chop.edu/calcbmi.php).

## Hormone assays

All hormone assays were performed using fresh samples, except for inhibin B assay that was performed on frozen samples.

### AMH

Serum AMH was determined using an enzyme-linked immunoassay specific for human AMH (EIA AMH/MIS®, Beckman-Coulter Co., Marseilles, France), as previously validated (12, 27). Intra- and inter-assay coefficients of variation were, respectively, 10.5% and 9.4% for a serum AMH concentration of 700 pmol/L (98 ng/mL), and 11.1% and 12.8% for a serum AMH concentration of 7 pmol/L (0.98 ng/mL). When serum AMH levels were undetectable, a value of 1 pmol/L (0.14 ng/mL), corresponding to the limit of quantification (functional sensitivity), was attributed.

### Inhibin B

Serum inhibin B was determined using an enzyme-linked immunoassay specific for human inhibin B (Inhibin B Gen II ELISA®, Immunotech Beckman-Coulter Co., Prague, Czech Republic). Intraassay coefficients of variation were 14% for a serum inhibin B concentration of 111 pg/mL, and 9.8% for 479 pg/mL. Inter-assay coefficients of variation were 14% for a serum inhibin B concentration of 12 pg/mL, and 7.7% for 210 pg/mL. When serum inhibin B levels was undetectable, a value of 7.2 pg/mL, corresponding to the limit of quantification (functional sensitivity), was attributed.

### Testosterone

Testosterone was determined in serum using an electro-chemiluminescent immunoassay (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) as described (12). Intra- and inter-assay coefficients of variation were 2.4% and 2.6%, respectively, for a mean testosterone concentration of 176 ng/dL (6.10 nmol/L) and 1.2% and 2.3% for a mean testosterone concentration of 455 ng/dL (15.78 nmol/L). When serum testosterone levels were undetectable, a value of 10 ng/dL (0.347 nmol/L), corresponding to the limit of quantification (functional sensitivity), was attributed.

### Gonadotropins

LH and FSH were determined using electro-chemiluminescent immunoassays (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) as described (27). The limits of quantification of both LH and FSH assays were 0.10 IU/L, according to the 2nd National Institute for Biological Standards and Control International Standard (NIBSC IS) 80/552 for LH and the 2nd World Health Organization International Reference Preparation (WHO IRP) 78/549 for FSH. Intra- and inter-assay coefficients of variation were 1.1% and 1.8%, respectively, for a mean LH concentration of 2.8 IU/L and 1.4% and 1.5% for a mean LH concentration of 16.9 IU/L. Intra- and inter-assay coefficients of variation were 1.0% and 4.2%, respectively, for a mean FSH concentration of 14.8 IU/L and 1.1% and 4.1% for a mean FSH concentration of 23.4 IU/L. When serum LH or FSH levels were undetectable, the value of the limit of quantification (functional sensitivity) was attributed.

### Clinical chemistry

Serum albumin and C-reactive protein (CRP) were measured using immunoturbidimetric assays in Cobas c501 (Roche), and hemoglobin was determined by a colorimetric method in Mindray BC-6800 Plus.

## Statistical analyses

Data distribution was assessed for normality using the Shapiro-Wilks test. Results are expressed as median and interquartile range. For comparisons of median SDS in patients at diagnosis with the theoretical median of 0 SDS in the reference population, we used the parametric one sample t test in case of normal distribution and, in the opposite case, the nonparametric Wilcoxon signed rank test was used. In the same way, depending on the distribution, paired t test or Wilcoxon matched-pairs signed rank test were used to compare between basal hormones and after 3 months of treatment in the individual follow-up of patients. The level of significance was calculated for groups ≥7 patients and set at $P < 0.05$. All statistical analyses were performed using GraphPad Prism version 9.4.1 for Windows (GraphPad Software, San Diego, CA, USA).

The calculation of the study sample size was performed to determine the proportion of patients with Sertoli cell dysfunction up to 36 months of follow-up in the longitudinal cohort of boys and adolescents diagnosed with hematopoietic malignancies. All of the patients of the longitudinal study who had clinical and hormonal assessments at diagnosis and after 3 months of chemotherapy were included in the present study. Since this is an interim study of the whole cohort, a specific sample size calculation was not performed.

## Ethical issues

Research was conducted in accordance with principles of the Declaration of Helsinki and local regulations on observational studies with human subjects. The study protocol was approved by the Institutional Review Board of the Ricardo Gutiérrez Children's Hospital, Buenos Aires (# CEI 14.07). All patients or their parents, as appropriate, provided informed consent.

# Results

## Characteristics of the study sample

Twenty out of 121 eligible patients were not included for the following reasons: 12 initiated chemotherapy treatment prior to the first hormonal evaluation, 7 had trisomy 21 and 1 had corpus callosum agenesis (Figure 1). From the 101 patients with hematopoietic malignancies included in the long-term follow-up cohort, a serum sample at 3 months was not available in 7 patients, who were therefore excluded. From the 94 patients analyzed in the present study, 70 (74.5%) were prepubertal. As expected, ALL represented the most frequent diagnosis, and boys with ALL were younger than those with AML or NHL; the analysis of the results of the whole study were made according to the pubertal status (Table 1). The median time between diagnosis and post-chemotherapy serum sample was 2.9 months (interquartile range: 2.6-3.3).

## Prepubertal boys

Serum levels of AMH, inhibin B and FSH were measured to assess the functional status of the gonadotrophin-Sertoli cell component of the HPT axis. Given that reference hormone levels change during childhood and puberty, we analyzed our results using SDS. Nonetheless, absolute hormone levels are provided in Supplementary Table 2. At diagnosis, serum levels of all 3 hormones were significantly below the median levels expected for the reference population, as analyzed using the Wilcoxon signed rank test: AMH (median -0.39 SDS, IQR -0.89 to 0.06, P<0.001), inhibin B (-0.39 SDS; IQR -0.91 to 0.23, P=0.035) and FSH (-0.65 SDS, IQR -0.94 to 0.20, P<0.001) (Figure 2). Serum levels below 0 SDS were observed in 70% of the boys for AMH, 64.6% for inhibin B and 83.8% for FSH. However, values below -2 SDS were found in only 1 patient for AMH and 2 boys for FSH. Altogether, these results suggested the existence of a mild impairment of the FSH-Sertoli cell axis function at diagnosis in prepubertal boys with hematopoietic malignancies. After 3 months of chemotherapy, there was an increase in median serum levels of all 3 hormones: AMH (-0.23 SDS, IQR -0.76 to 0.82), inhibin B (-0.06 SDS, IQR -0.94 to 0.20) and FSH (0.08 SDS, IQR -0.65 to 1.19). FSH levels were above 2 SDS in 11 patients (16.2%) at 3 months of treatment.

When analyzed by diagnosis, baseline AMH was decreased in patients with SR/IR-ALL (-0.45 SDS; IQR -0.85 to 0.06) but not in those with HR-ALL (-0.35 SDS; IQR -0.74 to 0.17) or NHL (-0.61 SDS; IQR -1.16 to -0.07) (Figure 3A). An increase to 0.45 SDS (-0.65 to 1.11 SDS) was observed in serum AMH after 3 months of chemotherapy in patients with SR/IR ALL, but no significant changes were seen in boys with HR-ALL or NHL (Figure 4A). Inhibin B was -0.27 SDS (-0.79 to 0.18) in SR/IR ALL, -0.51 SDS (-0.80 to 0.89) in HR-ALL and -0.96 SDS (-1.15 to 0.02) in NHL at diagnosis (Figure 3B). After 3 months of chemotherapy, inhibin B increased in patients with SR/IR ALL patients (0.05 SDS; IQR -0.63 to 0.71 SDS), but did not significantly change in boys with HR-ALL or NHL (Figure 4B). Regarding FSH, significantly decreased levels were observed in all groups: SR/IR-ALL (-0.55, IQR -0.91 to -0.19),

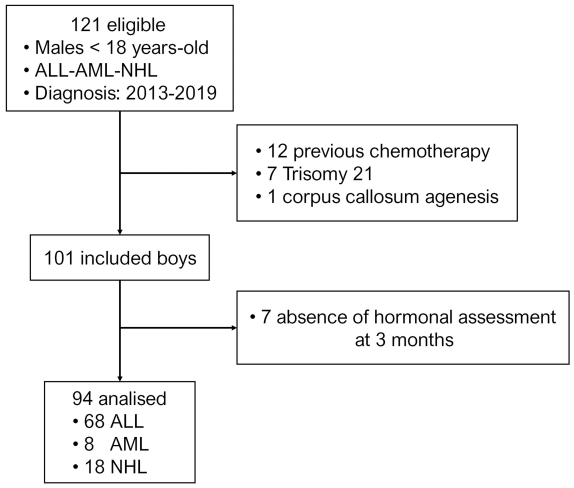


FIGURE 1
Flow-chart of patients included in the study.

HR-ALL (-0.67, IQR -1.10 to -0.24) and NHL (-0.85; IQR -1.28 to -0.52) (Figure 3C). After 3 months of chemotherapy, FSH increased to 0.12 SDS (-0.50 to 1.13) in patients with SR/IR-ALL and to 0.32 SDS (-0.69 to 3.10) in boys with NHL (Figure 4C).

Although our initial aim was to determine the immediate impact of chemotherapy in the first 3 months, we sought to determine whether the changes in hormone levels remained stable. Data shown in Supplementary Table 4 indicate that outcomes were overall stable until at least the 6$^{th}$ month of treatment.

## Pubertal boys

To assess the functional status of the pituitary-Sertoli cell axis, we used serum levels of FSH, inhibin B and AMH, whereas for the pituitary-Leydig cell axis we analyzed serum levels of LH and testosterone. Since reference hormone levels change according to Tanner stage, SDS were used for the analysis. Nonetheless, absolute hormone levels are provided in Supplementary Table 3.

At diagnosis, values significantly below the median levels expected for the reference population were seen for serum inhibin B (-0.90 SDS; IQR -2.01 to -0.14, P=0.019, one sample t test) and AMH (median -0.83 SDS, IQR -1.12 to -0.26, P=0.029, Wilcoxon signed rank test), whereas median FSH was within the reference range (-0.37 SDS, IQR -1.14 to 0.39, P=0.519, one sample t test). Serum inhibin was below 0 SDS in 80.0% of the boys and 4 patients (26.7%) had levels below -2 SDS, while AMH was below 0 SDS in 83.3% but none was below -2 SDS. After 3 months of chemotherapy, median inhibin B (-0.67 SDS; -1.87 to 0.13, P=0.010, one sample t test) and AMH (-0.55 SDS; -1.05 to 0.07, P=0.028, Wilcoxon signed rank test) remained below 0 SDS, with no significant changes compared to the basal state, whereas median FSH (2.19 SDS; 0.74 to 4.68, P<0.001, one sample t test) increased significantly to abnormally high levels. FSH was above +2 SDS in 13 patients (54.2%) (Figure 5A). Altogether, these results suggest that the Sertoli cell component is disrupted at diagnosis and the dysfunction persists after 3 months of chemotherapy, inducing an hypergonadotropic state.

The pituitary-Leydig cell axis showed decreased levels of testosterone (-1.00 SDS; -1.86 to -0.29, P=0.004, Wilcoxon signed rank test) with normal LH (0.49 SDS; -0.80 to 2.32, P=0.179, Wilcoxon signed rank test) at diagnosis. Serum testosterone was below 0 SDS in 83.3% of the patients and 3 patients (12.5%) showed serum testosterone below <2 SDS. After 3 months of chemotherapy, median testosterone increased significantly (1.15 SDS; -0.30 to 2.09, P=0.003, one sample t test), while LH showed median levels above 0 SDS (2.12 SDS; 0.94 to 3.63, P<0.001, one sample t test); LH was above +2 SDS in 13 patients (54.2%) (Figure 5B). These observations suggest that Leydig cell function was impaired at diagnosis and improved after 3 months of chemotherapy in association with high levels of LH.

TABLE 1 Characteristics of the study sample.

| Pubertal status | Diagnosis | Risk | n | % | Age (years) |
|---|---|---|---|---|---|
| Whole cohort | | | 94 | | 6.9 (0.5-17.6) |
| | ALL | SR/IR | 44 | 47% | 4.5 (1.5-16.8) |
| | | HR | 24 | 26% | 8.9 (0.5-16.4) |
| | AML | | 8 | 9% | 11.9 (1.8-14.5) |
| | NHL | | 18 | 19% | 8.8 (1.9-17.6) |
| Prepubertal | | | 70 | | 4.4 (0.5-14.3) |
| | ALL | SR/IR | 37 | 53% | 4.4 (1.5-14.3) |
| | | HR | 18 | 26% | 5.2 (0.5-10.9) |
| | AML | | 3 | 4% | 5.9 (1.7-7.8) |
| | NHL | | 12 | 17% | 6.4 (1.9-10.1) |
| Pubertal | | | 24 | | 13.7 (9.3-17.6) |
| | ALL | SR/IR | 7 | 29% | 13.5 (11.8-16.8) |
| | | HR | 6 | 25% | 14.9 (11.7-16.4) |
| | AML | | 5 | 21% | 13 (11.9-14.5) |
| | NHL | | 6 | 25% | 15.4 (9.3-17.6) |

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; NHL, non-Hodgkin lymphoma; SR, standard risk; IR, intermediate risk; HR, high risk.

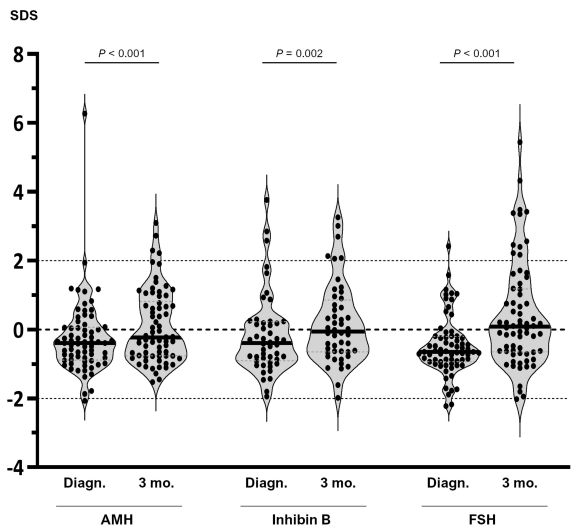


FIGURE 2
Serum levels of AMH, inhibin B and FSH in prepubertal boys with hematopoietic malignancies at diagnosis (Diagn.) and after 3 months (3 mo.) of chemotherapy. Values are expressed as standard deviation scores (SDS). Within each violin, the line represents the median. Comparisons between diagnosis and 3 months were analysed using the Wilcoxon matched-pairs signed rank test.

Individual assessments by diagnosis are shown in Supplementary Figures 2, 3. Statistical analyses were not possible due to the low number of observations in each subgroup. Data in Supplementary Table 5 indicate that changes were overall stable until 6 months of chemotherapy.

## General health status

In prepubertal boys, median serum hemoglobin and albumin were low at diagnosis and increased after 3 months of chemotherapy, whereas median C-reactive protein was high and normalized after 3 months (Supplementary Table 6). In pubertal patients too, hemoglobin and albumin were low and C-reactive protein was high at diagnosis. While hemoglobin did not significantly change at 3 months, albumin increased, and C-reactive protein decreased to normal levels. Altogether, these results suggest that the general health status was compromised at diagnosis and improved after 3 months of chemotherapy. The prevalence of boys in whom the hormonal status improved at 3 months was similar in those who showed a better health status, as reflected in C-reactive protein levels, and in those who did not (Supplementary Table 7).

# Discussion

The results of the present study show that, at diagnosis, a large proportion of prepubertal boys with ALL, AML or NHL had lower AMH, inhibin B and FSH concentrations compared to the reference population, reflecting an FSH-Sertoli cell axis dysfunction before any treatment was initiated. After 3 months of chemotherapy, all

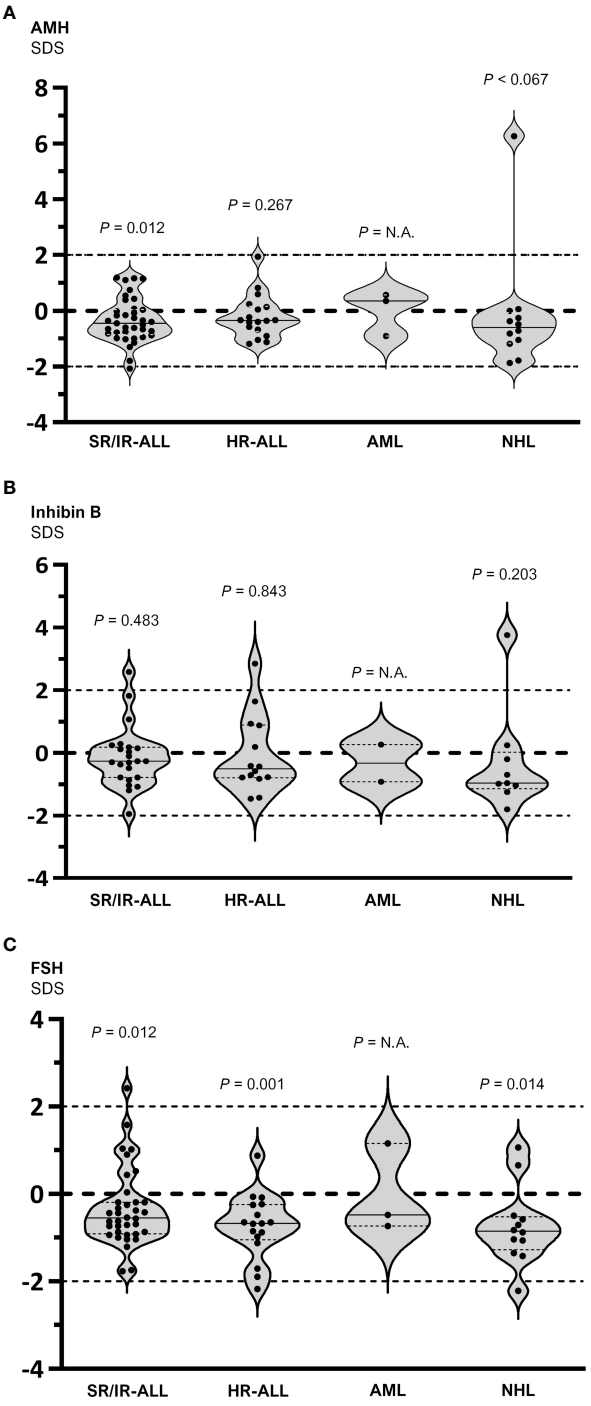


FIGURE 3
Serum hormone levels in prepubertal boys with hematopoietic malignancies grouped by diagnosis: standard/intermediate risk (SR/IR) and high risk (HR) acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and non-Hodgkin lymphoma (NHL). Values are expressed as standard deviation scores (SDS) and compared to the theoretical value of 0 SDS. In the group with AML, a statistical analysis was not applicable (N.A.) given the insufficient number of observations. Within each violin, the full line represents the median and the dotted lines the 25$^{th}$ and 75$^{th}$ centiles. **(A)** AMH: one sample t test was used for SR/IR and HR ALL, and Wilcoxon signed rank test for NHL. **(B)** Inhibin B: one sample t test was used for SR/IR and HR ALL, and Wilcoxon signed rank test for NHL. **(C)** FSH: a Wilcoxon signed rank test was used for SR/IR ALL, and a one sample t test for HR ALL and NHL.

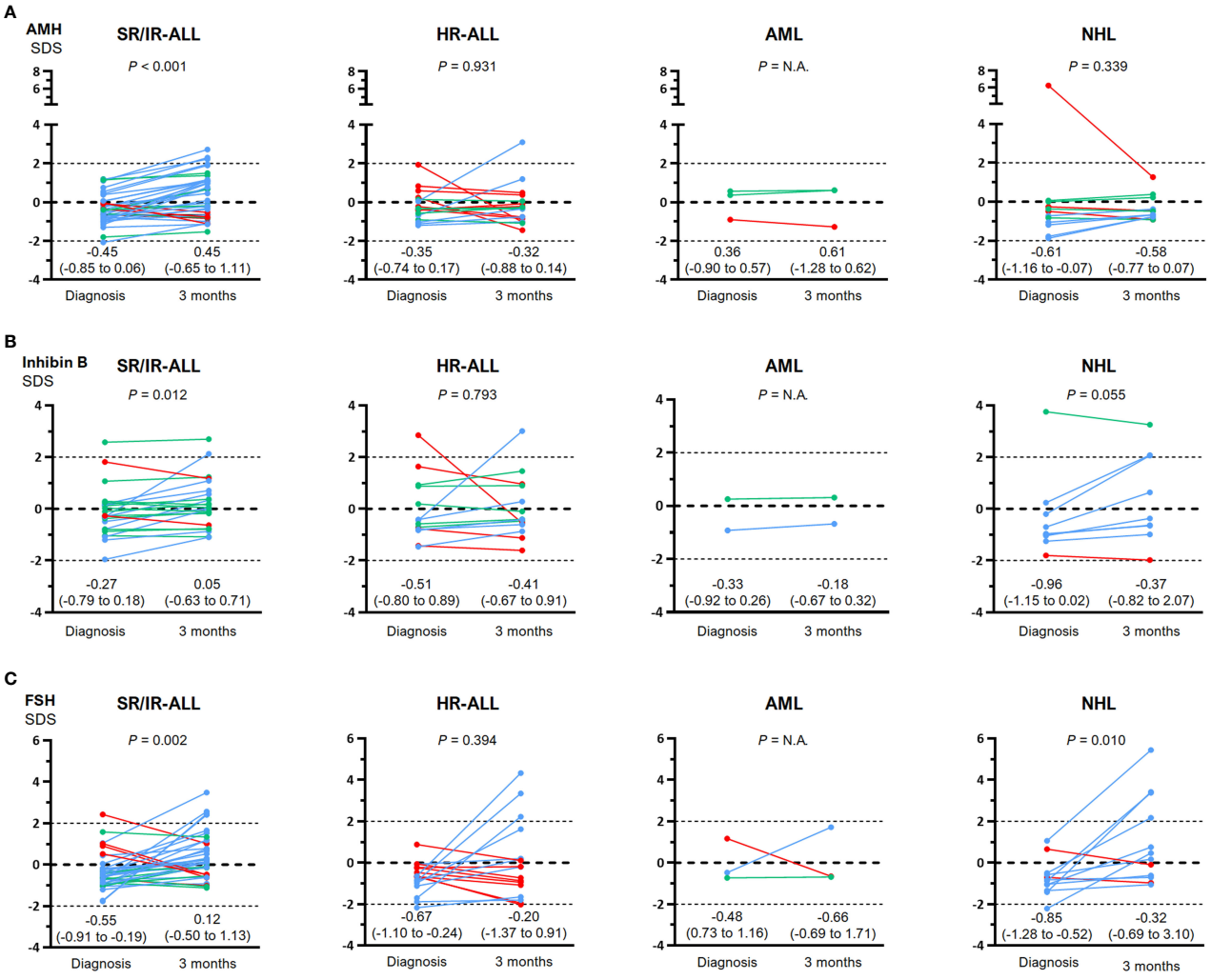


FIGURE 4
Comparison between serum hormone levels at diagnosis and after 3 months of chemotherapy in individual prepubertal boys with hematopoietic malignancies: standard/intermediate risk (SR/IR) and high risk (HR) acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and non-Hodgkin lymphoma (NHL). Values are expressed as standard deviation scores (SDS). Light blue lines indicate an increase, green lines denote no change and red lines, a decrease in hormone levels. **(A)**: AMH. For each individual, serum AMH was considered to increase or decrease when the difference between diagnosis and 3 months was >20% or <20%, respectively. Statistical analyses were performed using a paired t test for SR/IR and HR ALL, and Wilcoxon matched-pairs signed rank test for NHL. **(B)**: Inhibin B For each individual, serum inhibin B was considered to increase or decrease when the difference between diagnosis and 3 months was >25% or <25%, respectively. Statistical analyses were performed using paired t test for SR/IR and HR ALL, and Wilcoxon matched-pairs signed rank test for NHL. **(C)**: FSH. For each individual, serum FSH was considered to increase or decrease when the difference between diagnosis and 3 months was >5% or <5%, respectively. Statistical analyses were performed using Wilcoxon matched-pairs signed rank for SR/IR and HR ALL, and a paired t test for NHL. In all the boys with AML, statistical analyses were not applicable (N.A.) given the insufficient number of observations.

hormone concentrations increased, especially in the prepubertal boys with SR/IR-ALL. At pubertal age, boys with hematopoietic malignancies had lower AMH and inhibin B concentrations compared to the reference population for Tanner stage, with inappropriately normal FSH levels at diagnosis, reflecting a primary Sertoli cell dysfunction with inadequate gonadotrophin compensation. After 3 months of chemotherapy, inhibin B and AMH did not recover and FSH increased to high levels, suggesting a significant impairment of the Sertoli cell function. The LH-Leydig cell axis function was slightly impaired at diagnosis. After 3 months of chemotherapy, the increase in testosterone concomitantly with an elevation in LH to supraphysiological levels probably reflects a compensated Leydig cell impairment.

AMH and inhibin B are produced by Sertoli cells, the most active cell population during childhood. The low concentrations of these two peptides at diagnosis in prepubertal boys with leukemias or non-Hodgkin lymphoma could be due to primary Sertoli cell dysfunction. Our results in boys with hematopoietic malignancies are in line with a previous report in a large series of prepubertal males with various cancer types (30). Testicular dysfunction may be due to gonadal infiltration by tumor cells and/or by the action of inflammatory cytokines on gonadal tissue. Indeed, testicular infiltration has been extensively described in boys with leukemia (31–33), and CXCL12 expressed in Sertoli cells has been identified as part of a paracrine signaling system with the potential to sustain the migration and persistence of leukemic cells in the testis (34). The coexistence of

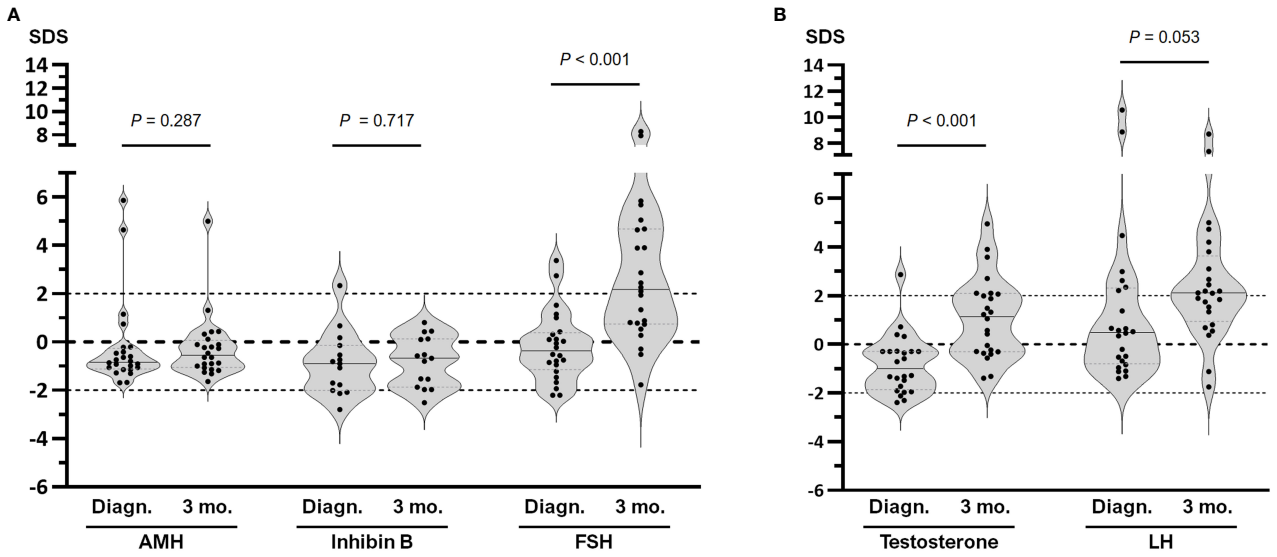


FIGURE 5
Hormone serum levels in pubertal boys with hematopoietic malignancies at diagnosis (Diagn.) and after 3 months of chemotherapy (3 mo.). **(A)** Pituitary-Sertoli cell axis, including AMH, inhibin B and FSH. **(B)** Pituitary-Leydig cell axis, including testosterone and LH. Values are expressed as standard deviation scores (SDS); comparison between Diagn. and 3 mo. were analysed using the Wilcoxon signed rank test for AMH, testosterone and LH, and paired t test for inhibin B and FSH. Within each violin, the line represents the median.

low FSH levels with Sertoli cells hypofunction could indicate a concomitant central hypogonadism in prepubertal boys, that is, a combined primary and central hypogonadism (24). Central hypogonadism can occur in response to a generalized disease that deteriorates the general condition. In our series of patients, low serum hemoglobin and albumin together with increased C-reactive protein at diagnosis favor the hypothesis of an affected general health status. However, the improvement in the hormone levels in a subset of patients without a clear recovery of the health status suggests the involvement of additional underlying mechanisms, for instance an effect of chemotherapy on gonadal infiltration by leukemic cells.

In boys with congenital central hypogonadism, low inhibin B and AMH levels indicate a Sertoli cell hypoplasia secondary to pituitary gonadotropin deficiency reflecting a long-term insufficient FSH activity on Sertoli cell proliferation and function (15). Conversely, acquired central hypogonadism results in a less significant effect on Sertoli cell biomarkers, likely due to the fact that sufficient FSH activity induced normal Sertoli cell proliferation during fetal and neonatal periods leading to an adequate mass of Sertoli cells, as reflected by normal testicular volume in most cases (35).

The increase in FSH, AMH and inhibin B after 3 months of treatment in the prepubertal boys was unexpected given that chemotherapy is believed to have gonadotoxic effects, especially when alkylating agents are used (36). Our results suggest an improvement of the FSH-Sertoli cell axis during the initial phases of chemotherapy, which coincides with an improvement in the biomarkers of general health status. Noteworthy, testicular function, as reflected by serum AMH and inhibin B, improved in patients with SR/IR ALL but not in those with HR-ALL, AML or NHL. The lower number of patients with HR-ALL, AML and NHL may deter the detection of a statistically significant difference. Alternatively, the more significant increase in FSH levels in patients with SR/IR-ALL could underlie the better response in AMH and inhibin B. This is the first study to report an improvement of the HPT axis function in the initial months of chemotherapy in boys with hematopoietic malignancies.

In boys diagnosed during puberty, the endocrine function of the testis was affected both in the interstitial (Leydig cell) and the seminiferous tubule (Sertoli cell) compartments, in agreement with previous findings in all cancer types (30) and in a smaller study including specifically adolescents with leukemia or lymphoma (37). Testicular function in pubertal boys with Hodgkin disease was described to be more adversely affected by chemotherapy than in prepubertal boys long time ago (38). Although chemotherapy regimens have changed, it is interesting to point out that this pioneering observation focused on germ cell depletion during pubertal maturation. Here, we add the value of Sertoli cell biomarkers. The production of AMH and inhibin B differ in pubertal boys: AMH is produced exclusively by Sertoli cells whereas inhibin B is secreted as a cooperative action of Sertoli and germ cells. The low concentrations of these two peptides at diagnosis could be due to a primary Sertoli cell dysfunction (low AMH) and germ cell damage (low inhibin B). The coexistence of inappropriately normal FSH levels with low inhibin B in pubertal boys could indicate, like in prepubertal boys, a combined hypogonadism. The same analysis applies to the LH-Leydig cell axis, where low testosterone coexisted with inappropriately normal LH. Conversely, after 3 months of chemotherapy, the behavior of the two compartments differed. Despite an increase of both FSH and LH to supraphysiological levels, Inhibin B and AMH did not recover whereas median testosterone exceeded 1 SDS. This suggests a dissociated hypogonadism, with a compensated Leydig cell activity and an insufficient seminiferous tubule function. A more toxic effect of chemotherapy on the germ cell population (39, 40) could explain

the insufficient increase in serum inhibin B. Alternatively, Sertoli cells could be primarily affected. In fact, there is an FSH-induced peak of Sertoli cell proliferation in the initial phases of puberty (41), and proliferating cells are more sensitive to most chemotherapy agents. As regards the LH-Leydig cell loop, it could also be influenced by a Sertoli-cell paracrine dysregulation. Indeed, impaired Sertoli cell function, either primary or due to germ cell depletion, may result in increased activin secretion and signaling on neighboring Leydig cells, thus leading to decreased testosterone production requiring higher LH stimulation (42).

In conclusion, the HPT axis is impaired at diagnosis in boys with hematopoietic malignancies. There seems to be a combined hypogonadism, with a primary testicular dysfunction possibly due to gonadal infiltration by tumor cells and/or the effect of inflammatory factors on the gonad, and a concomitant functional central hypogonadism that could be due to an impaired overall health. The HPT axis function improves during the initial 3 months of chemotherapy, with certain nuances according to age and testicular compartment, concomitantly with the general health state.

## Data availability statement

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

## Ethics statement

The studies involving human participants were reviewed and approved by Comité de Ética en Investigación, Hospital de Niños Ricardo Gutiérrez. All patients or their parents, as appropriate, provided informed consent.

## Author contributions

RG, RR, GD, LA, MGR and IB conceived the study design; JD, SP, LB, MB, MER, MEG, MS, LM, CF and PB collected clinical and laboratory data; JD, SP, LB, RR and RG analyzed the data; JD, RR and RG drafted the manuscript; All authors contributed to the article and approved the submitted version.

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## Conflict of interest

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

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.1135467/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Risk group definition based on age, white blood cell count, cytogenetics, response on peripheral blood at day 8, on bone narrow at days 15 and 33 and flow cytometric-minimal residual disease according to ALL IC-BFM 2009 protocol. BM: bone marrow, FCM-MRD: Flow cytometric-minimal residual disease, M1: <5% bone marrow blasts, M2: 5-25% bone marrow blasts, M3: >25% bone marrow blasts, PB: peripheral blood, WBC: white blood cells.

SUPPLEMENTARY FIGURE 2
Comparison of pituitary-Sertoli cell hormone levels at diagnosis and after 3 months of chemotherapy in individual pubertal boys with hematopoietic malignancies: standard/intermediate risk (SR/IR) and high risk (HR) acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and non-Hodgkin lymphoma (NHL). Values are expressed as standard deviation scores (SDS, median and interquartile range). Light blue lines indicate an increase, green lines denote no change and red lines, a decrease in hormone levels. **(A)** AMH. For each individual, serum AMH was considered to increase or decrease when the difference between diagnosis and 3 months was >20% or <20%, respectively. **(B)** Inhibin B. For each individual, serum inhibin B was considered to increase or decrease when the difference between diagnosis and 3 months was >25% or <25%, respectively. **(C)** FSH. For each individual, serum FSH was considered to increase or decrease when the difference between diagnosis and 3 months was >5% or <5%, respectively. Statistical analyses were not performed given the insufficient number of observations in each subgroup.

SUPPLEMENTARY FIGURE 3
Comparison of pituitary-Leydig cell hormone levels at diagnosis and after 3 months of chemotherapy in individual pubertal boys with hematopoietic malignancies: standard/intermediate risk (SR/IR) and high risk (HR) acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and non-Hodgkin lymphoma (NHL). Values are expressed as standard deviation scores (SDS, median and interquartile range). Light blue lines indicate an increase, green lines denote no change and red lines, a decrease in hormone levels. **(A)** Testosterone. For each individual, serum testosterone was considered to increase or decrease when the difference between diagnosis and 3 months was >5% or <5%, respectively. **(B)** LH. For each individual, serum LH was considered to increase or decrease when the difference between diagnosis and 3 months was >3% or <3%, respectively. Statistical analyses were not performed given the insufficient number of observations in each subgroup.

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# Depressive and anxiety symptoms, and neural correlates of reward and punishment anticipation in female athletes with amenorrhea

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**Objective:** Studies in estrogen deficiency states such as primary ovarian insufficiency and Turner syndrome suggest that estrogen status may be an important modulator of mood and emotions. In this study we compared depressive and anxiety symptoms between adolescent and young adult female oligo-amenorrheic athletes (AA) and eumenorrheic females (EM), and explored structural, and functional changes in related brain areas during reward processing, a behavioral construct that is altered in depression and anxiety.

**Methods:** We included (i) 24 AA participating in ≥4 hours/week of aerobic exercise or running ≥20 miles/week for ≥6 months in the preceding year, with lack of menstrual cycles for ≥3 months within at least 6 preceding months of oligo-amenorrhea, OR in premenarchal girls, absence of menses at >15 years), and (ii) 27 EM aged 14-25 years. Participants completed the Beck Depression Inventory-II (BDI-II), State and Trait Anxiety Inventory (STAI), and Mood and Anxiety Symptoms Questionnaire (MASQ). Structural MRI and brain activation during a functional MRI (fMRI) task that probes reward and punishment processing was examined in a subset of 10 AA and 23 EM.

**Results:** Median (IQR) age and BMI of AA and EM groups were 20.6 (19.0-22.6) vs. 20.6 (19.2-23.7) years, p=0.6 and v 20.3 (18.8-21.5) vs. 21.9 (19.6-23.5) kg/m2, p=0.005, respectively. While groups did not differ for BDI-II scores, AA had higher anhedonic depression MASQ scores (p=0.04), and STAI (p=0.03) scores vs. EM. In the fMRI subset, AA had higher caudate volumes vs. EM [F(1, 29)=9.930, p=0.004]. Lower activation observed in the right caudate during reward anticipation in AA compared with EM (p=0.036) suggests blunted reward processing in the striatum in estrogen deficient states.

**Conclusion:** Athletes with amenorrhea had higher depressive and anxiety symptomatology compared to eumenorrheic young women. Exploratory analyses demonstrated increased caudate volumes and decreased caudate activation during reward processing in athletes with amenorrhea suggesting that estrogen may play a role in reward processing.

KEYWORDS

estradiol, adolescent, anxiety, depression, reward and punishment processing

# 1 Introduction

Approximately 11.3% of adolescents in the United States between ages 12-17 years have one episode of major depression in the past year (1) and about 31.9% develop anxiety disorders between the ages of 13-18 years with a higher prevalence in females (2). These disorders are a major cause of disability among adolescents and young adults, and a significant risk factor for suicide, which is the third leading cause of death for adolescents aged 15-19 years (3). Understanding the factors that impact mood and anxiety in adolescents may provide novel direction regarding the management of these disorders.

Emerging evidence indicates that estrogen may play a significant role in modulating mood. In particular, estrogen deficiency states such as menopause and Turner syndrome have been associated with a higher risk of depression and anxiety compared to the general population (4, 5). Exercise without appropriate nutritional fueling can result in low energy availability, even in normal-weight athletes; this disrupts the hypothalamic-pituitary-gonadal (HPG) axis, leading to functional hypothalamic amenorrhea (FHA) and low estrogen levels (6). While numerous reports have demonstrated a positive effect of exercise on depression and anxiety (7, 8), the role of estrogen deficiency resulting from physical activity in young athletes with amenorrhea on mood and anxiety has not been studied in detail. To our knowledge, only one study has evaluated mood issues in athletes with respect to their menstrual status and reported a trend for a higher prevalence of bipolar disorders or major depressive disorders in 23% of runners who had absent menses (amenorrhea) versus 0% runners with regular menses (eumenorrhea) (9). Similarly, an increased prevalence of depressed mood has been reported in FHA similar to that in organic amenorrhea, suggesting that estrogen deficiency might be a common factor in both groups driving mood issues (10, 11). Of relevance, we recently reported improvement of trait anxiety with estrogen replacement in girls with FHA (12). However, studies evaluating depression and anxiety in athletes who have estrogen deficiency and are amenorrheic are limited.

One of the hallmarks of mood disorders is abnormal reward and punishment processing that is often associated with structural and functional changes in reward-related brain regions, most prominently the striatum, including the caudate, putamen and the nucleus accumbens (NAc) (13–15). Of note, the ability to anticipate reward is often blunted in depression and is a core behavioral construct that is frequently tested in functional neuroimaging studies examining depression. In general, these studies have shown reduced brain activation in the caudate while anticipating and receiving a reward among adolescents with depression (16). The anticipatory response to punishment is less well known in depression. However, induced anxiety has been reported to increase striatal activation to both rewards and punishment, suggesting anxiety symptoms may impact incentive processing generally (17). Based on these findings we designed a study to compare depressive and anxiety symptoms in adolescent and young adult athletes with irregular or absent menses (oligo-amenorrhea) versus athletes and non-athletes with regular menses (eumenorrhea). Further, we compared a subset of these subjects with respect to structural (volumetric) features of the striatum as well as functional striatal activation using a monetary incentive paradigm that probes a behavioral construct altered in depression/anxiety. We hypothesized that estrogen deficiency would negate the beneficial effects of exercise, and that compared to eumenorrheic females (EM), female athletes with oligo-amenorrhea (AA) would have greater symptoms of depression and anxiety. Additionally, we hypothesized that oligo-amenorrheic athletes would exhibit structural changes in the striatum (caudate, putamen and NAc) and have altered neural activation during reward/punishment anticipation when compared to the EM group. This novel study is the first of its kind to evaluate the relationship of menstrual status on mood in adolescent and young adult female athletes, and underlying neural correlates.

# 2 Methods

## 2.1 Participants

Study participants were recruited by advertising in institutional recruitment forums, sports medicine treatment centers and physician practices. The study included 51 right-handed females between ages 14-25 years with BMI between the 10th-90th

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percentiles: 24 AA, 14 EA and 13 NA. Participants were grouped by menstrual status into two categories: one with oligo-amenorrhea (AAs only, n=24) and the other with regular menses or eumenorrhea and no medical issues, EM (EA, and NA taken together, n=27). Athletes were defined as those participating in ≥4 hours/week of aerobic exercise or running ≥20 miles/week for a period of ≥6 months in the preceding year. NA did not participate in team sports and exercised <2 hours/week. Oligo-amenorrhea was defined as lack of menstrual periods for ≥3 months within at least 6 preceding months of oligo-amenorrhea, or in premenarchal girls, absence of menses at >15 years. Exclusion criteria included (a) conditions other than excessive exercise causing loss of periods, including pregnancy, polycystic ovary syndrome and other conditions of hyperandrogenism, thyroid dysfunction (unless euthyroid on medications for at least 3 months), primary ovarian insufficiency, and hyperprolactinemia, and (b) any contraindications to estrogen use such as an increased predisposition for thromboembolism, underlying liver disease, and a personal or first-degree family history of an estrogen dependent cancer. All participants (or their parents) provided written informed consent to the protocol, which was approved by our Institutional Review Board. Participants below 18 years also provided signed assent for study participation.

## 2.2 Procedure

Following a screening visit, participants completed questionnaires, had a structural MRI scan and completed a monetary incentive delay task in the scanner. Twenty-three AA and 26 participants from the eumenorrheic group, EM, completed questionnaires to assess their mood. Depressive, anxiety, and anhedonic symptoms were assessed using the Beck Depression Inventory-II (BDI-II) (18), the State-Trait Anxiety Inventory (STAI) (19, 20), and the Mood and Anxiety Symptoms Questionnaire (MASQ) (21) respectively. Imaging data were obtained in 10 AA and 23 EM participants. The majority of EM participants (except for 9) were brought in during the proliferative phase of their cycle, which was determined based on their last menstrual period. Estradiol levels were available for 9 AA and 23 EM participants. Estradiol was assessed by chemiluminescence (Beckman Coulter, Fullerton, CA; sensitivity 20 pg/mL; intraassay CV 2.0-4.2%). We used menstrual status (rather than an estradiol level) as a surrogate of estrogen status given that estradiol levels vary across the menstrual cycle and are dependent on cycle phase, and they do not necessarily reflect the chronicity of estrogen exposure. Further, standard estradiol assays are not always reliable at low levels.

## 2.3 MRI procedure

Please see the Supplement for detailed information on MRI procedures, data quality assurance, preprocessing, and statistical analysis.

## 2.4 Monetary incentive delay task

The monetary incentive delay task is designed to measure neural responses to anticipation and receipt of rewards and penalties and has been demonstrated to successfully recruit the striatum in healthy individuals (22). Previous studies using this task have revealed reduced striatal activation to reward anticipation in depressed/anhedonic individuals and increased striatal activation to both reward and punishment in anxious individuals, respectively, compared with healthy adults (17, 23, 24) making it well suited for the present study. At the onset of each trial, participants were presented with a visual cue (0.5 sec) indicating the potential outcome (reward: +$; penalty: –$; no incentive: 0$). After a variable inter-stimulus interval (2.25–3.75 sec), a red target square was briefly presented (0.15 sec). After a second variable delay (2.4–3.9 sec), visual feedback (1.25 sec) based on trial outcome (reward, penalty, or no change) was displayed and a variable intertrial interval ensued (1.5-4.5 sec). Participants were instructed to respond to the square by pressing a button as quickly as possible and that speed determines the probability of success. In order to match task difficulty across participants, the 70th percentile of each participant's reaction time during a practice session was defined as the individual's reaction time threshold for success. Practice session was identical to the design described above except that no feedback was displayed. In the reward condition, successful trials were associated with monetary gains ($1.98 to $2.31), whereas unsuccessful trials led to no-change. In the penalty condition, successful trials were associated with no-change, whereas unsuccessful trials were associated with monetary penalties (-$1.85 to -$2.17). No-incentive trials always ended with no-change feedback. The task included five blocks of 24 trials (8 reward, 8 penalty, and 8 no-incentive trials).

## 2.5 Imaging data acquisition

MRI data were acquired on a 3T scanner (Siemens Medical Systems, Iselin, NJ) equipped with a 32-channel head coil. fMRI data were acquired using a T2*-weighted multiband echo-planar imaging sequence (multiband factor = 3, repetition time = 1.65 sec).

## 2.6 Imaging data analyses

Both structural and functional images were processed using fMRIPrep 20.2.1 (25, 26), which is based on Nipype 1.5.1 (27, 28). Detailed information is provided in the Supplement.

### 2.6.1 Structural data analyses

The T1-weighted (T1w) image was intensity bias-corrected and skull-stripped. Brain surfaces were reconstructed using recon-all (FreeSurfer 6.0.1). Intracranial volume (ICV) was calculated to correct for inter-individual differences in total brain size. All estimated volumes of *a priori* structures were exported to SPSS for statistical analyses.

### 2.6.2 Functional data analyses

Preprocessing: The following preprocessing steps were performed – skull-strip, susceptibility distortion correction, slice time correction, spatial normalization, spatial smoothing, and automatic removal of motion artifacts using ICA-AROMa (Prium 2015). Additional motion criteria for exclusion were applied to the fMRI data (see Supplement).

First-level Analyses: First five volumes (8.25 sec) were discarded as dummy scans from the beginning of the task and the onset times were adjusted accordingly. First-level of single-subject fMRI data from all runs were implemented using a general linear model (GLM) in SPM. For each run, the following regressors were included in the model-reward cue, penalty cue, no-incentive (neutral) cue, successful reward feedback, unsuccessful reward feedback, successful no penalty feedback, unsuccessful penalty feedback and no-change feedback (no-incentive condition). In addition, mean time-series from the cerebrospinal fluid (CSF) and white matter (WM) masks generated from fmriprep, target, errors (i.e., when the button was pressed before the target presentation) and inter-stimulus intervals (ISIs) were included as covariates of no-interest. Each event was constructed with a hemodynamic response function, modeled using a gamma function, convolved with onset times of events and stimulus duration. A high-pass temporal filter (128 sec/0.008 Hz) was also applied to the model. Contrast maps were constructed for reward anticipation (reward vs. neutral cue) averaged across runs. The analyses were focused only on reward anticipation here due to small sample size. These contrast maps were used in region of interest (ROI)-based statistical analyses to test *a priori* hypotheses as well as for whole-brain main effects analysis evaluating brain regions affected by the task.

## 2.7 Statistical analyses

Our primary hypotheses were to investigate the effect of oligo-amenorrhea on depressive and anxiety symptoms. We also explored associated neural correlates of reward and punishment processing in the striatum (specifically caudate, putamen, and nucleus accumbens (NAc)). To this end, we focused our analyses on parsing group differences between those with eumenorrhea (EA + NA) and oligo-amenorrhea (AA). All analyses were performed in SPSS (version 22).

### 2.7.1 Demographics and clinical questionnaires

Normality of data was determined using the Shapiro-Wilk test. Given that most data were not normally distributed, non-parametric tests (Mann-Whitney U test) were used for comparisons.

### 2.7.2 Structural data analyses

Striatal volumes for each subject from Freesurfer outputs were exported to SPSS for analyses. To test striatal volumes, a Group (oligo-amenorrheic/eumenorrheic) x ROI (Caudate, Putamen, NAc) x Hemisphere (Left, Right) repeated measures ANOVA was run controlling for age and Intracranial volume (ICV). Significant interactions were followed by individual *post-hoc* tests.

### 2.7.3 Functional data analyses

To investigate the neural correlates of reward and punishment anticipation and to validate the task, a one-sample t-test was conducted across all subjects. Cluster correction at $p < 0.05$ family-wise error (FWE) with an initial voxel forming threshold of $p < 0.001$ was utilized. ROI Analyses: To test *a priori* hypotheses that oligo-amenorrhea would affect reward-related activation in the striatum, we created left and right anatomical NAc, caudate, and putamen ROIs from the FSL Harvard-Oxford Subcortical Atlas using a 40% probability threshold. For each subject, parameter estimates from each of the ROIs were extracted from reward and penalty anticipation contrast maps and were entered into SPSS (version 22). Throughout the analyses, data were inspected for the presence of outliers. Values that exceeded three times the inter-quartile range (the difference between the third and first quartile) of mean parameter estimates were deemed to be outliers and were further investigated to identify if they were due to motion, registration error, or other sources of artifacts. If no problems could be identified and corrected, outlier data points were removed. After inspection for outliers, a repeated measures ANOVA with Group (amenorrheic/eumenorrheic as a between-subject factor and Hemisphere (Right/Left) and Valence (Cue Reward – Cue Neutral, CR_CN/Cue Punishment – Cue Neutral, CP_CN) as within-subject factors was run for each ROI to investigate effects of amenorrhea on reward and punishment anticipation.

# 3 Results

## 3.1 Demographics and clinical questionnaires

Table 1 summarizes participant characteristics in the two groups. AA had lower BMI and were significantly older at menarche compared with the eumenorrhea group (t=-2.85, p=0.005; t=2.65, p=0.006, respectively). As expected, AA had higher anxiety (measured by STAI Trait scores, t=2.01, p=0.03) and depressive symptomatology (higher anhedonic depression measured by MASQ scores, t=2.04, p=0.04) and lower estradiol levels (t=-2.02, p=0.004) compared with those with eumenorrhea (see Table 2).

## 3.2 Structural analyses

Striatal ROI: Group (oligo-amenorrhea, eumenorrhea) x ROI (Caudate, Putamen, NAc) x Hemisphere (Left, Right) repeated measures ANOVA controlling for ICV and age revealed a significant ROI x Group [$F(1,2)=4.6$, $p=0.014$, $\eta 2=0.14$] and a main effect of Group [$F(1,29)=5.49$, $p=0.026$, $\eta 2=0.16$], but no effects of ROI or Hemisphere. Further *post-hoc* analyses adjusted for Bonferroni correction revealed that AA had higher caudate volumes compared to the eumenorrheic group [$F(1, 29)=9.930$, $p=0.004$, $\eta^2=0.26$]. However, no significant differences were observed for

TABLE 1 Participant characteristics by menstrual groups.

| | Oligo-amenorrheic group (n=24) Median (IQR) | Eumenorrheic group (n=27) | P value | Effect size Cohen's D |
|---|---|---|---|---|
| **Age (years)** | 20.6 (19.0-22.6) | 20.6 (19.2-23.7) | 0.578 | 0.14 |
| **BMI (kg/m$^2$)** | 20.3 (18.8-21.5) | 21.9 (19.6-23.5) | **0.005** | 0.8 |
| **Percent median BMI** | 93.9 (90.0-100.0) | 101.8 (91.7-108.6) | **0.008** | 0.27 |
| **Activity (hours/week) *** | 8.5 (6.7-11.3) | 4.3 (0.4-9.9) | **0.011** | 0.56 |
| **Age of menarche (years) *** | 13.0 (12.5-15.0) | 12 (12-13) | **0.006** | 0.74 |

Median and IQR (25$^{th}$-75$^{th}$) Mann-Whitney U Test was used for the overall p value; P values <0.05 are bolded. *Data only available for this variable in 23 oligo-amenorrheic participants.

putamen [F(1, 29)=0.28, p>0.5, $\eta^2$=0.009] or NAc volumes (F(1, 29) =2.81, p=0.11, $\eta^2$=0.09; see Figure 1].

## 3.3 Functional monetary incentive delay analyses

Two participants (two EA) were excluded as they had >10% of trials classified as motion outliers based on motion criteria mentioned in the Data Quality section in the Supplement. Whole brain analysis of reward and punishment anticipation across all subjects was conducted to validate the task. Consistent with other studies, significant activations were observed in the bilateral striatum, mid-cingulate and orbitofrontal cortex (see Supplement Figures S1, S2 and Tables S1, S2).

### 3.3.1 Caudate

Group (oligo-amenorrhea, eumenorrhea) x Hemisphere (Left, Right) x Valence (CR_CN/CP_CN) repeated measures ANOVA in the caudate revealed a significant Group x Hemisphere effect [F (1,29)=5.61, p=0.025, $\eta^2$=0.16). *Post-hoc* analyses adjusted for Bonferroni correction revealed that this effect was mainly driven by differences in the right caudate, with lower activation observed in the AA group (p=0.036, $\eta^2$=0.14) during both reward and punishment anticipation. No significant differences were observed in the left caudate (p=0.29, $\eta^2$=0.04; Figure 2).

### 3.3.2 Nucleus accumbens

A Group x Valence x Hemisphere interaction was observed [F (1,29)=4.7, p=0.038, $\eta^2$=0.14], but further *post-hoc* analyses revealed no significant effects.

### 3.3.3 Putamen

No main effects or interactions were observed in the putamen during reward or punishment anticipation.

## 3.4 Sensitivity analyses

To ensure that structural and functional differences between oligo-amenorrheic and eumenorrheic individuals were not driven by depression/anxiety, physical activity, we repeated the analyses controlling for these variables.

Controlling for depression and anxiety scores: Anhedonic depression and anxiety scores data were not available for 1 oligo-amenorrheic and 1 eumenorrheic individual. The higher caudate volume and lower caudate activation to reward/punishment anticipation observed in AA compared to the EM group remained significant even after controlling for anhedonic depression/anxiety scores [Caudate volume - F(1, 25)=7.234, p=0.013, $\eta^2$=0.22; Caudate functional activation – F(1, 25)=4.778, p=0.038, $\eta^2$=0.16].

Controlling for physical activity (Average hours/week for the past year): Physical activity data were not available for 1 oligo-

TABLE 2 Questionnaires and hormones by menstrual groups (to be removed, added to manuscript).

| | Oligo-amenorrheic group (n=23) | Eumenorrheic group (n=26) | P value | Effect Size Cohen's d |
|---|---|---|---|---|
| **BDI-II** [a] | 4 (0-13.8) | 1.5 (0-3) | 0.19 | 0.453 |
| **STAI** [a] | 32.5 (27.0-51.0) | 27 (25-36.3) | **0.03** | 0.583 |
| **MASQ (Anhedonic Depression)** | 51 (42.0-64.0) | 42.0 (35.3-48.3) | **0.04** | 0.583 |
| **Estradiol (pg/ml)**[b] | 24.4 (16.5-38) | 52.5 (32.5-107.0) | **0.004** | 0.835 |

Median and IQR (25th-75th). Mann-Whitney U Test was used for the overall p value. BDI-II, Beck Depression Inventory-II; STAI, State-Trait Anxiety; MASQ, Mood and Anxiety Symptom Questionnaire; P values <0.05 are bolded.
[a]Data only available for these variables in n = 22 (oligo-amenorrheic group).
[b]Data only available in 9 oligo-amenorrheic and 23 eumenorrheic participants.

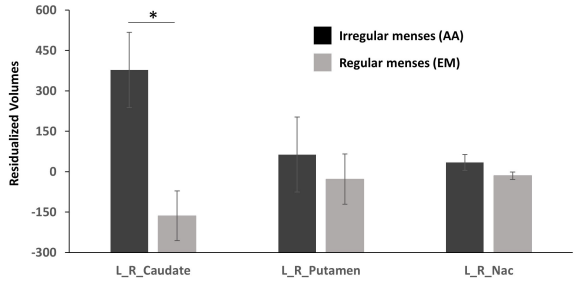


FIGURE 1
Residualized volumetric (corrected for age and ICV) differences in the caudate, putamen, and nucleus accumbens (NAc) between participants with oligo-amenorrhea and eumenorrhea. Error bars indicate standard error. *p < 0.05.

amenorrheic individual. AA had higher caudate volumes compared to the EM group after controlling for physical activity [F(1, 27)=6.060, p=0.021, $\eta^2$=0.18]. However, after controlling for physical activity, the decreased activation in the right caudate during reward and punishment anticipation in AA reached significance only at a trend level [F(1,270 = 3.76, p = 0.063, $\eta^2$=0.12], suggesting physical activity may play a small role in this effect.

Sensitivity analyses for age at menarche (as age at menarche was different between groups) and BMI (as a proxy for nutritional status) are provided in the Supplement.

## 4 Discussion

Our study adds to the sparse literature on mood and anxiety and related structural and functional reward-related changes in oligo-amenorrheic athletes. Consistent with our hypothesis that estrogen deficiency is associated with mood and anxiety alterations; depressive and anxiety symptoms were significantly higher in female athletes with oligo-amenorrhea compared to young women with eumenorrhea. Further, bilateral caudate volume was higher in AA compared with the eumenorrheic group even after adjusting for age and intracranial volume. We also found that AA

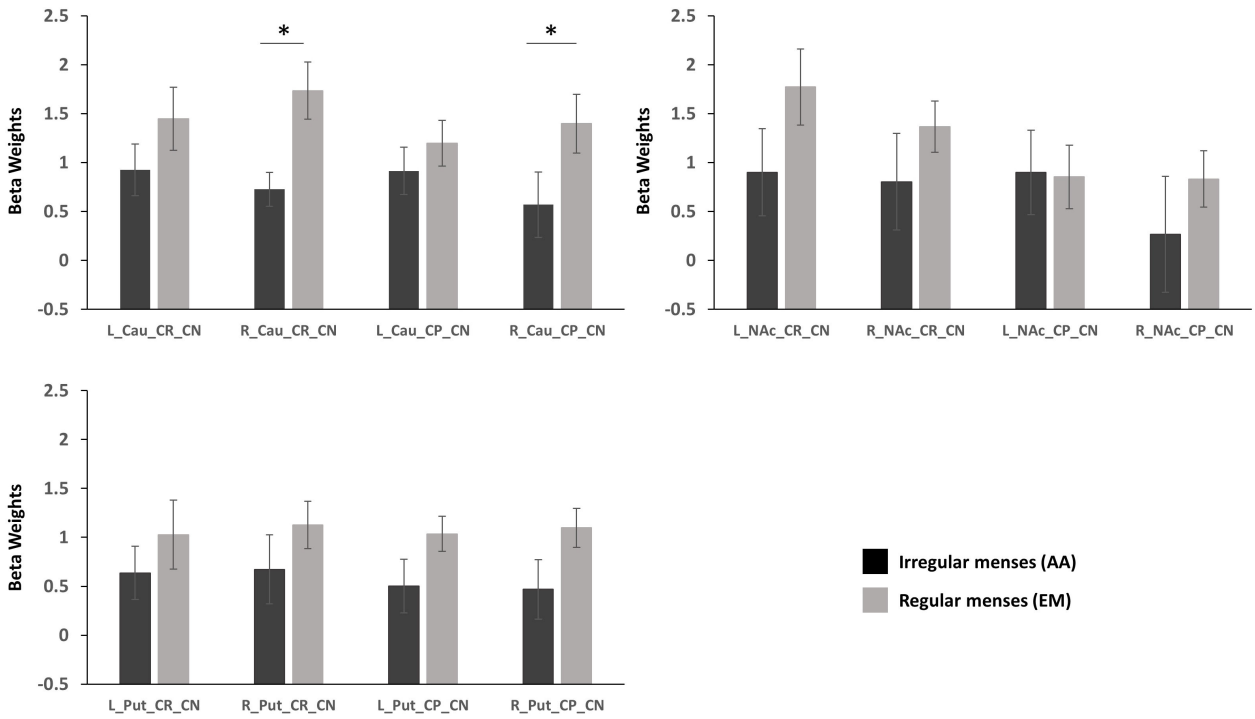


FIGURE 2
Standardized parameter estimates extracted from functional ROIs during reward anticipation (Cue Reward – Cue Neutral, CR_CN) and punishment activation (Cue Punishment – Cue Neutral, CP_CN) in the caudate (Cau), putamen (Put), and nucleus accumbens (NAc) in participants with oligo-amenorrhea and eumenorrhea. Error bars indicate standard error. *p < 0.05.

had decreased activation in the right caudate during anticipation of reward and punishment in the monetary incentive delay task, suggesting that estrogen status may play a role in modulating mood and anxiety. These findings persisted even after we controlled for possible confounders.

Effects of gonadal hormones on mood have been demonstrated in ovariectomized mice, who show symptoms of increased anxiety and reluctance to venture into the center of an open area to seek out food (29) as well as depressive behavior (30). Interestingly, such behaviors normalize after estrogen replacement (30, 31). Women with estrogen deficiency (e.g., post-menopause, Turner syndrome, primary ovarian insufficiency) have a higher prevalence of mood alterations and anxiety compared with the general population (4, 5, 32). Further, functional imaging studies in normally menstruating adult women have described a correlation between estradiol levels and blood oxygen level dependent (BOLD) signal activation in the amygdala-hippocampal region following a reward, indicating that gonadal steroids may play a role in modulation of reward processing (33). Importantly, in a prior study, we showed that hypogonadal adolescent girls with anorexia nervosa (AN) and FHA have an improvement in trait anxiety following estrogen replacement (12).

Consistent with these reports, in the current study, AA exhibited higher scores for anhedonic depression and anxiety compared to the eumenorrheic group. Athletic amenorrhea is a form of FHA, which is characterized by increased depressive symptoms and difficulty dealing with stressors. These individuals tend to display dysfunctional attitudes such as perfectionistic behavior, extra attention to the judgments of others and unrealistic expectations compared to eumenorrheic individuals (10, 11). In one study of 21 healthy controls, 18 amenorrheic women with AN, and 13 normal-weight women with FHA, women with AN and FHA had higher scores for anxiety and depressive symptomology based on the Hamilton Rating Scale for Anxiety (HAM-A) and Depression (HAM-D) than the healthy controls (34). It is important to note that, although depressive and anxiety symptoms were higher in the group with oligo-amenorrhea, mean scores in our study did not reach the level of clinical significance for a diagnosis of anxiety or depression. Exercise, by virtue of being a mood elevator, may have offered some protection to these individuals.

The finding that AA demonstrated increased volume of the caudate region compared to eumenorrheic participants suggests that estrogen may play an important role in volume reductions in specific brain areas during puberty. A longitudinal pediatric neuroimaging study by Giedd et al. indicated that gray matter in the frontal lobe increased linearly during pre-adolescence with maximum size noted before puberty, followed by a decline post-adolescence resulting in a net decrease in volume. Gray matter volume in the parietal lobes followed a similar pattern for changes in volume, except that the slopes were steeper pre- and post-adolescence. This structural change in both lobes peaked one year earlier in females than males, suggesting that maturational volume reductions in these regions may be potentially driven by the influence of estrogen (35). Of note, Lepage et al. evaluated developmental brain morphology in adolescents with Turner syndrome (TS), a condition characterized by a lack of endogenous estrogen. This study compared 30 girls with TS with 21 age-matched healthy female controls, and showed that hypoestrogenic girls with TS have significantly higher caudate volume (similar to findings in athletes with oligo-amenorrhea in our study) suggesting that this may result from delayed maturational involution linked to the lack of estrogen during adolescence (36).

Rodent studies indicate that estrogen has dopamine agonist actions and promotes dopamine secretion in the striatum and thereby improves reward sensitivity (37, 38). Functional neuroimaging studies in humans have yielded similar findings. During the mid-follicular phase of the menstrual cycle, estradiol levels positively correlate with brain activity in the amygdala and hippocampal complex during reward anticipation (33). In a more recent study, Macoveneau et al. examined reward processing in 58 women randomized to GnRH analogues (which block sex steroid production) and placebo (39). Compared to participants on placebo, the GnRH analogue group showed reduced activation in the amygdala to monetary gains suggesting that sex steroids may modulate reward processing (39). Of note, these participants also had increased depressive symptoms. Similarly, estrogen replacement in perimenopausal women resulted in increased response in the striatum during reward anticipation relative to placebo (40). Previous studies have also demonstrated hypoactivation to gains as well as penalties in patients with depression compared with healthy individuals (24). Our finding of decreased activation in the caudate regions for reward and punishment anticipation (functional MRI task) is in line with these findings and with our hypothesis that estrogen deficiency may blunt the response to reward processing.

The main limitation of our study is the small number of participants with MRI scans. This resulted in the eumenorrheic athlete and non-athlete groups being combined, as they both have regular menses (and also because these two groups were similar for STAI and MASQ scores). The significance of reduced caudate activation in AA vs EM ($p=0.025$) did not survive multiple corrections applied for 3 ROIs ($p = 0.05/3 = 0.017$), possible driven by small sample size. However, it is important to note that our results are characterized by moderate effect sizes. A few participants could not be included due to technical difficulties with MRI processing and image acquisition, and it will be important to replicate our findings in a larger sample. The second limitation relates to the relatively normal scores of anhedonic depression and anxiety in study participants, indicating no increase in a clinical diagnosis of depression or anxiety, notwithstanding higher mean STAI and MASQ anhedonic depression scores in oligo-amenorrheic vs. eumenorrheic women. Thus, higher mean scores on these questionnaires do not necessarily translate to a clinical diagnosis of anxiety or depression (known to be associated with structural brain changes). This was further corroborated by our sensitivity analyses that showed the significances for caudate functional and structural alterations persisted even after controlling for anhedonic depression and anxiety scores. Similarly, controlling for physical activity and menarchal age did not change our results for structural

differences between groups. However, the reduced caudate activation in the AA group was significant only at a trend level after controlling for physical activity, suggesting that exercise, by virtue of being a mood elevator, may have offered some (albeit a small) protection to these individuals. The lack of significance may have been due to the small sample size in our exploratory analyses. Lack of data regarding caloric intake and the use estrogen status based on menstrual status (as estradiol levels were not available in all individuals and do not necessarily reflect the chronicity of estrogen exposure as they are cycle phase dependent) are other limitations that need to be addressed in future studies. Although our data provide important preliminary evidence that estrogen status may play a role in brain structure and function, we cannot determine causality as these are cross sectional studies. These findings underscore the need to identify young athletes with estrogen deficiency and to evaluate the implications of these findings on psychiatric endpoints. From a clinical perspective, identifying comorbidities related to estrogen status may help propose early interventions involving improved diet, reduced activity and psychological counseling and may help improve outcomes.

## 5 Conclusion

Oligo-amenorrheic athletes have greater anxiety and depressive symptoms compared with young women with eumenorrhea. Oligo-amenorrheic athletes also have increased caudate volumes, which may be related to delayed maturational decrease in cortical volumes seen in estrogen deficiency states. In addition, relative to eumenorrheic athletes and non-athletes, oligo-amenorrheic athletes demonstrate blunted responses to rewards. These findings require validation in larger studies.

## Data availability statement

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

## Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board MGH. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

PK and SN analyzed the data, CB, PK and SN interpreted the data. CB, PK, SN, and MM were contributors in writing the manuscript. FP, KA, KE, DP, and MM edited and reviewed and edited the manuscript. CB and MM conceptualized and obtained the funding for the study. All authors read and approved the final manuscript.

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## Conflict of interest

Over the past 3 years, DP has received consulting fees from Albright Stonebridge Group, Boehringer Ingelheim, Compass Pathways, Engrail Therapeutics, Neumora Therapeutcis former BlackThorn Therapeutics, Neurocrine Bioscences, Neuroscience Software, Otsuka Pharmaceuticals, Sunovion Pharmaceuticals, and Takeda Pharmaceuticals; honoraria from the Psychonomic Society for editorial work and Alkermes, and research funding from NIMH, Dana Foundation, Brain and Behavior Research Foundation, Millennium Pharmaceuticals. In addition, he has received stock options from Compass Pathways, Engrail Therapeutics, Neumora Therapeutics former BlackThorn Therapeutics, and Neuroscience Software. Over the past 3 years, MM has received consulting fees from Sanofi and Abbvie, and served on the scientific advisory board for Abbvie and Ipsen. No funding from these entities was used to support the current work, and all views expressed are solely those of the author.

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

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2023.976050/full#supplementary-material

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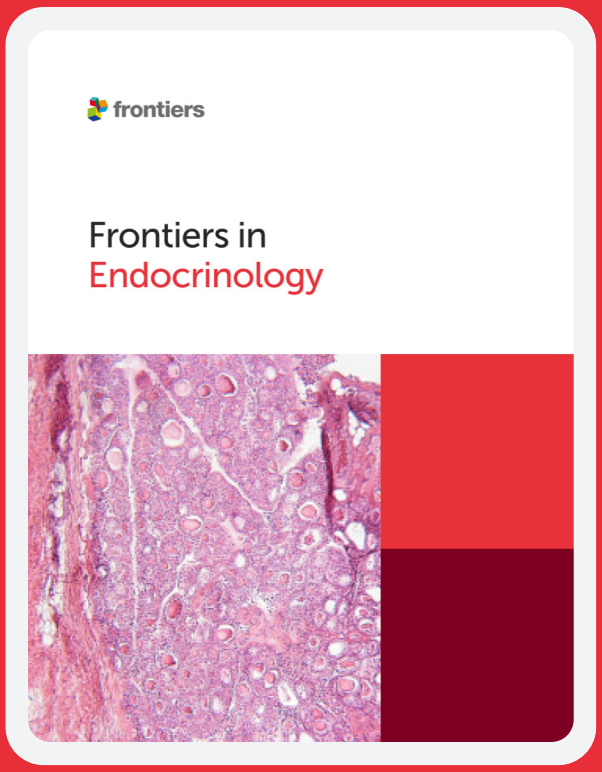


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