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# Genetic, epigenetic and molecular landscaping of puberty

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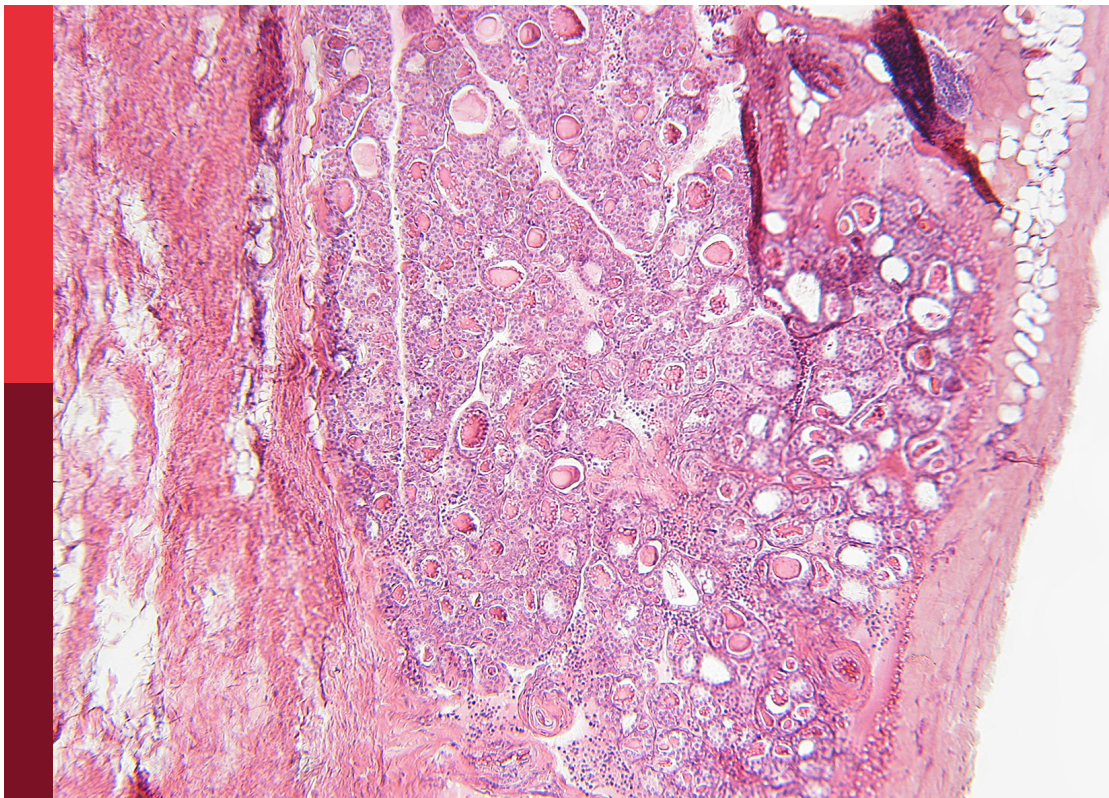

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# Genetic, epigenetic and molecular landscaping of puberty

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# Editorial: Genetic, epigenetic and molecular landscaping of puberty

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KEYWORDS

**puberty, central precocious puberty (CPP), delayed puberty onset, congenital hypogonadotropic hypogonadism, Klinefelter syndrome, epigenetics**

Editorial on the Research Topic
Genetic, epigenetic and molecular landscaping of puberty

The genomic landscape of puberty is a rapidly progressing area of pediatric endocrinology in which there have been major developments in our understanding over recent years. Given the importance of genetic, epigenetic, and molecular mechanisms in the regulation of puberty, this Research Topic aimed to collect new studies in this subject, from diverse fields including central precocious puberty, delayed puberty, hypogonadotropic hypogonadism, puberty in Klinefelter syndrome and Silver Russell syndrome, and environment-gene interactions in puberty

The article "*Pubertal timing in children with Silver Russell syndrome (SRS) compared to those born small for gestational age (SGA)*" presented an observational study of the pubertal and auxological features of patients with SRS as compared to patients with SGA alone. The authors, Patti et al., concluded that timing of puberty is affected in patients with SRS regardless of postnatal weight increase, and that puberty is earlier in patients with maternal uniparental disomy of chromosome 7 as compared to loss of methylation of chromosome 11p15.

In "*Dealing with Brain MRI Findings in Pediatric Patients with Endocrinological Conditions: Less Is More?*" the authors, Baldo et al., tackled a difficult clinical scenario of neurological imaging in children with pubertal disorders. Of particular challenge is the finding of incidental abnormalities on MRI brain scans in patients with central precocious puberty, such as arachnoid or pineal cysts, and the authors describe monitoring and follow up for these and other MRI findings in this group of patients.

The review article "*MKRN3 role in regulating pubertal onset: the state of art of functional studies*" summarizes and discusses some of the recent approaches developed to predict makorin ring finger protein 3 (MKRN3) functions and its involvement in pubertal development (Palumbo et al.). Loss-of-function mutations in this gene, in fact, represent the most commonly known genetic cause of central precocious puberty (CPP) but its role in pubertal onset control is not completely known.

In the work "*Navigating Disrupted Puberty: Development and Evaluation of a Mobile-Health Transition Passport for Klinefelter Syndrome*" the authors face the difficult problem of transition to adult care of patients with Klinefelter syndrome (Dwyer et al.). In particular they developed and tested a digital transition passport that was found to be usable, understandable, and had high ratings for actionability.

Faienza et al. provided a comprehensive review entitled "*Genetic, epigenetic and environmental influencing factors on the regulation of precocious and delayed puberty*" (5). In this article, the authors present the complex interaction of genes with environmental factors regulating pubertal timing. Indeed, defects in genes encoding kisspeptin receptor (*KISS1R*), Makorin Ring Finger Protein 3 (*MKRN3*) and Delta-like 1 homolog (*DLK1*), have been implicated in central precocious puberty, whereas mutations in a growing number of genes, such as *FGFR1*, *GNRHR*, *HS6ST1* and many others, contribute to delayed puberty. In addition to genetic defects, the authors discuss the role of epigenetics (DNA methylation and microRNAs, miRNAs) in the onset of puberty. Finally, Faienza et al. highlight the emerging involvement of endocrine disrupting chemicals, as environmental factors, in regulating pubertal initiation.

In the research article "*Correlation Analysis of Genotypes and Phenotypes in Chinese Male Pediatric Patients with Congenital Hypogonadotropic Hypogonadism*" Wang et al. studied the medical records of 125 Chinese male patients aged 0–18 years with congenital hypogonadotropic hypogonadism (CHH). The authors collected the clinical characteristics, the hormonal measurements, and the genetic defects of the participants through whole-exome sequencing, and performed a correlation analysis of genotypes and phenotypes. Importantly, they also found 15 new CHH-related genes, compared to previously published studies. Finally, Wang et al. concluded that cryptorchidism, micropenis and the genetic defects are *sine qua non* factors for early and accurate diagnosis of CHH in children and adolescents, and further discussed the long-term follow up of these patients.

In the article "*Integrated analysis of proteomics and metabolomics in girls with central precocious puberty*", the results of proteomics and metabolomics in serum samples from girls with CPP are presented in an attempt to identify potential biomarkers (Li et al.). Bioinformatic analyses led to the identification of 134 differentially expressed proteins in girls with CPP with 71 upregulated and 63 downregulated proteins and the identification of 103 differentially expressed metabolites, including 42 upregulated and 61 downregulated metabolites. By performing network analysis of integrated proteomics and metabolomics, the authors revealed lipid metabolic pathways that may be involved in pubertal development in girls.

The review article "*Genetic architecture of self-limited delayed puberty and congenital hypogonadotropic hypogonadism*" examines the distinction between self-limited delayed puberty and congenital hypogonadotropic hypogonadism (Vezzoli et al.). The authors provide an updated overview of the genetics behind these two conditions and discuss the advantages and disadvantages of genetic analysis, particularly since the introduction of next generation sequencing, to effectively distinguish between these two conditions.

Together, these articles demonstrate the fantastic complexity of the genetic and epigenetic control of puberty, and the importance of the interaction of molecular, genomic and clinical aspects in the etiology of pubertal disease. Understanding of the genetic and epigenetic mechanisms driving pubertal disorders can benefit diagnosis and therapeutic management for patients with these conditions, and also open up new avenues for basic science exploration to promote identification of important molecular mechanisms and associated pathways. Digital technologies, whether radiological tools or the use of mobile phones for health passports can also revolutionize patient care. But amongst all this new discovery, the basics of clinical care, such as precise phenotyping of patients to aid diagnosis and attendance to the patients' holistic and psychosocial needs, remain vital to support best outcomes for patients with disorders of puberty.

## 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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# Dealing With Brain MRI Findings in Pediatric Patients With Endocrinological Conditions: Less Is More?

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Neuroimaging is a key tool in the diagnostic process of various clinical conditions, especially in pediatric endocrinology. Thanks to continuous and remarkable technological developments, magnetic resonance imaging can precisely characterize numerous structural brain anomalies, including the pituitary gland and hypothalamus. Sometimes the use of radiological exams might become excessive and even disproportionate to the patients' medical needs, especially regarding the incidental findings, the so-called "incidentalomas". This unclarity is due to the absence of well-defined pediatric guidelines for managing and following these radiological findings. We review and summarize some indications on how to, and even if to, monitor these anomalies over time to avoid unnecessary, expensive, and time-consuming investigations and to encourage a more appropriate follow-up of brain MRI anomalies in the pediatric population with endocrinological conditions.

**Keywords: incidentaloma, growth hormone deficiency, central precocious puberty, incidental radiological finding, follow-up**

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## INTRODUCTION

The widespread use of brain magnetic resonance imaging (MRI) has put at our disposal incredibly accurate images that give us the chance to improve our diagnostic competence. However, this widespread use of neuroimaging has led to a remarkable increase of incidental radiological findings, the so-called "incidentalomas", which may generate interpretation uncertainty for radiologists and clinicians (1, 2). Here we describe two clinical cases to exemplify this context.

## CLINICAL SCENARIO 1

A boy was referred for short stature at the age of 5 years. His height was -2.3 standard deviation score (SDS), his growth rate was -2.6 SDS, and his bone age was 3 years. His previous medical history was unremarkable. Two stimulation tests were performed with arginine and insulin, and peak values of growth hormone (GH) were pathological (<8 ng/mL) in both, supporting the diagnosis of GH deficiency (GHD). No other pituitary deficits were found on laboratory tests. A brain MRI under sedation was

performed before starting treatment, highlighting the presence of "pituitary stalk interruption syndrome". An annual MRI follow-up was suggested, and the patient underwent a brain MRI every year for 7 consecutive years, through which the radiological finding remained stable.

## CLINICAL SCENARIO 2

A girl was referred for precocious puberty at the age of 7 years. On physical examination, Tanner stages B2, Ph2, and A2-3 were observed. Her growth rate was +6 SDS, and her bone age was 9 years. A pelvic ultrasound showed pubertal changes in the uterus and ovaries, and a GnRH stimulation test showed a pathological level of LH peak (> 5 ng/mL) and an LH/FSH ratio >1, confirming the diagnosis of central precocious puberty (CPP). A brain MRI showed the presence of an arachnoid cyst in the parietal lobe, with dimensions of 2 x 3 cm, not compressing nor close to the ventricular system. An annual MRI follow-up was suggested. The patient underwent a brain scan for 4 consecutive years, through which the radiological finding did not show any variation.

## DISCUSSION

Brain MRI is mandatory in many pediatric endocrinological conditions to detect anatomic anomalies and rule out neoplastic lesions (3, 4).

In two of the most frequent conditions in pediatric endocrinology, GHD (prevalence 1:4.000-1:10.000) and CPP (prevalence 1:5.000-1:10.000) (5), brain MRI is requested once stimulation tests confirm the clinical diagnosis (3, 4). Nevertheless, other rare endocrine conditions (such as Cushing syndrome, hyperprolactinemia, gigantism, central hypo- and hyperthyroidism, and diabetes insipidus) unquestionably require a brain MRI in their diagnostic work-up.

In GHD, brain MRI may show characteristic anatomic pituitary abnormalities that can explain the endocrine disorder (such as anterior pituitary dysplasia, pituitary stalk interruption syndrome [PSIS], Rathke cleft cyst [RCC], empty sella, etc.), but can also detect possible tumoral lesions (such as craniopharyngioma or Langerhans cell histiocytosis) (6).

In CPP, the international guidelines state that neuroimaging is mandatory in all males and in females under six years of age since both these groups have a higher chance of presenting brain lesions causing early pubertal development (such as hamartoma, astrocytoma/glioma, germinoma) (7–9). Conversely, MRI is still controversial in females aged 6-8 years: the incidence of cranial abnormalities in this age group is minimal (10–12), even if a possible late cancer diagnosis cannot be excluded by clinical and biochemical parameters only (13).

In both GHD and CPP, identifying anomalies and malformations during brain MRI, including "incidentalomas", is not infrequent (9, 14). Starting from the clinical scenarios we described, we realized that unrelated brain anomalies, incidentalomas, and anatomic anomalies represent a real problem in the pediatric population. Thus, we asked ourselves whether it was necessary to routinely monitor all these findings after their identification or not, based on the current medical literature. Avoiding unnecessary tests on pediatric patients is not just a matter of cost, both for the health system and the patients' families, but also of physical and psychological stress for the patients and their families. The image acquisition process for brain MRI lasts from 20 to 30 minutes, and procedural sedation is usually needed in children younger than 7 years of age. Furthermore, unnecessary investigations and tests may postpone the medical evaluation of other patients in real need of consult.

We performed a narrative review of the literature, which was limited to the pediatric population, to identify the most common brain abnormalities in patients with endocrinological conditions who require an MRI scan. We then conducted subsequent research to determine the current management for every specific MRI abnormal finding. In doing so, we decided to include exclusively papers that described cohorts of patients, excluding single case reports or international guidelines on the matter. For rare conditions, if more than one cohort study was identified, we reported the ones with the largest population. The selection of papers was limited to the English language. The following three electronic databases were searched: Pubmed, Scielo, and Scopus. As search string, we used a combination of the condition's name plus pediatric/children plus management/guidelines/follow up or, alternatively, the condition's name plus pediatric/children plus MRI/magnetic resonance. We also searched for pediatric guidelines referring to the overall topic of brain incidentalomas *via* the database above reported.

Remarkably, despite the increasing relevance of the matter, we found out that there was a general lack of guidelines on incidentalomas and MRI brain abnormalities in the pediatric population. While the medical literature has vastly discussed this topic in the adult population, only a few studies currently support pediatricians' decisions on managing these conditions. For example, a 2011 paper by the Endocrine Society formulated some practical guidelines for managing pituitary incidentalomas in adults but clearly stated that they do not apply to the pediatric population (15). On the other hand, most papers on the pediatric population either describe small sample populations or analyze only a single subtype of incidentaloma without considering the remaining anomalies, missing the big picture. The works by Souteiro et al. and Shareef et al. represent the two most extensive studies (2, 16). The first one identified 41 incidentalomas in a cohort of pediatric patients who underwent brain imaging for various reasons, primarily headache. Pituitary hypertrophy was the most common finding (29.3%), followed by arachnoid cysts (17.1%), pituitary adenomas (14.6%), and RCC (12.2%). Remarkably, 56.1% of these patients underwent a radiological reevaluation, but none of them presented dimensional progression (2). The second one described 31 incidental lesions, among which RCC was the most frequent (67.7%), followed by cystic pituitary lesions (19.4%) and microadenomas (12.9%). Only 5 patients had a radiological reevaluation, and lesion growth was never documented (16).

According to the available evidence, we divided the radiological findings into two main groups:

a) **Findings with definite or possible clinical or anatomical relevance**, i.e., conditions involving the sellar region that might affect the endocrine system function. These include:

- *Adenohypophysis hypoplasia*
- *Pituitary stalk interruption syndrome (PSIS)*
- *Ectopic neurohypophysis*
- *Empty sella, complete or partial*
- *Rathke cleft cysts (RCC)*
- *Pituitary adenomas*
- *Craniopharyngiomas*

Overall, these findings have been identified more frequently in GHD than CPP (3, 17). *Arnold Chiari type I malformation*, i.e., the descent of the cerebellar tonsils through the foramen magnum, cannot be included in this group since it does not affect the sellar region. Still, it often has clinical relevance for the patient (18, 19).

Only three findings can potentially grow over time in this category: RCC, pituitary adenomas, and craniopharyngiomas (20–22). There is evidence that craniopharyngioma may progress from a longstanding RCC *via* a transitional stage of extensive squamous metaplasia (23).

The remaining lesions cannot progress and thus do not need radiological follow-up. For example, in the first clinical scenario, no further MRIs should have been performed in a patient with PSIS.

b) **Findings without any relevance that lack a correlation with the endocrinological condition under study**. This group contains the so-called "incidentalomas", such as:

- *Arachnoid cysts*
- *Epiphyseal cysts*
- *Choroid plexus cysts*
- *Vascular anomalies*
- *Increased hypophyseal volume*

Various studies have followed these findings throughout the years with subsequent MRI (6). However, due to the lack of standardized protocols on how to, and even if to, monitor them, and to a general misunderstanding on their ability to evolve or not, it is not infrequent that these lesions can be troublesome to deal with, leading to a relevant number of pointless tests.

Among incidentalomas, only arachnoid cysts may be worth further radiological evaluations since they can grow to a large size if the retention of cerebrospinal fluid progresses through time. However, a radiological follow-up is recommended only in the presence of a large cyst or if the lesion is located close to the ventricular system, posing a risk of hydrocephalus (24). Therefore, in the second clinical scenario we described, no other exams were needed.

We propose a summary table to manage the most frequent brain anomalies found in pediatric patients affected by GHD and CPP that is now in use in our Institute (**Table 1**) (2, 18, 24–59). This table is provided only as a guide and does not give absolute indications.

Overall, there are a few elements that we would like to remark on.

First, all the indications reported in the table refer to brain MRI findings that are not causing any neurological

**TABLE 1** | Suggested management of the most frequent brain anomalies found in pediatric patients affected by growth hormone deficiency (GHD) and central precocious puberty (CPP).

| MRI Finding | Management and follow-up | Reference |
|---|---|---|
| ***Findings with definite or possible clinical or anatomical relevance*** | | |
| Adenohypophysis hypoplasia | Baseline laboratory tests<br>Not require follow-up imaging studies | (25, 26) |
| Pituitary stalk interruption syndrome (PSIS) | Baseline laboratory tests<br>Not require follow-up imaging studies | (27, 28) |
| Ectopic neurohypophysis | Baseline laboratory tests<br>Not require follow-up imaging studies | (29, 30) |
| Empty sella, complete or partial | Baseline laboratory tests<br>Repeat laboratory tests after 24-36 months (in the absence of clinical manifestations) | (31, 32) |
| Rathke cleft cyst (RCC) | → *Symptomatic:* MRI every year for 5 years after the surgical removal of the cyst + Laboratory tests<br>→ *Asymptomatic (when >5 mm):* MRI at 1, 3, 5 years + Laboratory tests | (33–35) |
| Pituitary adenoma | → *Symptomatic:* Surgical or medical therapy + Follow-up (laboratory test, MRI, visual field evaluation) according to the oncological guidelines<br>→ *Asymptomatic:*<br>- Macro (≥10 mm): laboratory tests + visual field evaluation + MRI at 6 months and then every year<br>- Micro (≥5 mm): laboratory tests + MRI every year for the first 3 years and every 1-2 years<br>- Micro (<5 mm): MRI not required | (36–38) |
| Craniopharyngiomas | Follow-up after surgery ± radio/chemotherapy according to the oncological guidelines | (39–42) |
| Arnold-Chiari type I | Neurosurgical consultation | (18, 43–45) |
| ***Incidentalomas*** | | |
| Arachnoid cysts | MRI should be repeated only in case of large cysts or localization at risk of hydrocephalus | (24, 46, 47) |
| Pineal cysts | MRI should be repeated only with cysts >14 mm and/or with an abnormal radiological pattern or clinical symptoms | (45, 48–51) |
| Choroid plexus cysts | Not require follow-up imaging studies or specific medical management | (52–54) |
| Vascular anomalies | Neurosurgical consult | (36, 55–57) |
| Increased hypophyseal volume | Not require follow-up imaging studies or specific medical management | (2, 58, 59) |

*Laboratory tests include: IGF-1, cortisol, prolactin, FSH and LH, estradiol/testosterone, TSH and FT4.*

manifestation. In the presence of signs or symptoms suggestive of endocranial hypertension, such as headache, vomit, and arterial hypertension with bradycardia, an MRI evaluation is always warranted, regardless of a history of previous brain findings incidental or not.

Another critical element is that the pituitary gland volume should always be compared to the normal dimensions per age and sex of the patient. In our personal experience, we deal with various inappropriate endocrinological referrals due to misinterpretation of pituitary gland dimensions, especially coming from centers lacking pediatric radiologists. The most typical context is the identification of an enlarged pituitary gland in a teenager who underwent brain MRI because of recurrent headaches but in which no possible underlying elements were found. Therefore, before starting a complete examination of the pituitary gland functionality with a laboratory test and scheduling a follow-up brain MRI, we suggest evaluation of the initial imaging study by a radiologist with expertise in pediatric neuro-imaging.

A third clarification must be made on vascular anomalies. In this case, a neurosurgical consult is necessary because their follow-up and management depend primarily on their correct classification. For example, while cerebral developmental venous anomalies are managed conservatively (56), arteriovenous malformations are usually treated surgically or endoscopically because of their high risk of rupture in the pediatric population (55). A similar approach must also be chosen for asymptomatic Arnold-Chiari type 1 malformations since the timing of their radiological follow-up is currently discussed among specialists, even if the management is known to be conservative (60).

## CONCLUSIONS

The purpose of this manuscript is not to underestimate the possible relevance of incidental MRI findings in the diagnostic process of GHD and CPP. Our goal was to highlight that, based on the current literature, most of the follow-up brain MRIs are probably not required in these incidentalomas and that only a few neuroimaging findings are worth subsequent investigations.

## DATA AVAILABILITY STATEMENT

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

## AUTHOR CONTRIBUTIONS

FB, MM, and FM performed the literature search and wrote the manuscript. EB critically reviewed the manuscript for important intellectual content. GT conceptualized the paper and reviewed and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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# Correlation Analysis of Genotypes and Phenotypes in Chinese Male Pediatric Patients With Congenital Hypogonadotropic Hypogonadism

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Congenital hypogonadotropic hypogonadism (CHH) can be divided into Kallmann syndrome (KS) and normosmic HH (nHH). The clinical and genetic characteristics of CHH have been studied in adults, but less in pre-adults. The medical records of patients with CHH in our gonad disease database from 2008 to 2020 were evaluated. In total, 125 patients aged 0 to 18 years were enrolled in our study. KS patients had a higher incidence of micropenis compared with nHH (86.2% vs. 65.8%, p=0.009), and 7 patients (5.6%) had hypospadias. Among the 39 patients with traceable family history, delayed puberty, KS/nHH, and olfactory abnormalities accounted for 56.4%, 17.9%, and 15.4%, respectively. In total, 65 patients completed the hCG prolongation test after undergoing the standard hCG test, and the testosterone levels of 24 patients (22.9%) were still lower than 100 ng/dL. In 77 patients, 25 CHH-related genes were identified, including digenic and trigenic mutations in 23 and 3 patients, respectively. The proportion of oligogenic mutations was significantly higher than that in our previous study (27.7% vs. 9.8%). The most common pathogenic genes were *FGFR1*, *PROKR2*, *CHD7* and *ANOS1.* The incidence rate of the genes named above was 21.3%, 18.1%, 12.8% and 11.7%, respectively; all were higher than those in adults (<10%). Most mutations in CHH probands were private, except for W178S in *PROKR2*, V560I in *ANOS1*, H63D in *HS6ST1*, and P191L and S671L in *IL17RD*. By analyzing family history and genes, we found that both *PROKR2* and *KISS1R* may also be shared between constitutional delay of growth and puberty (CDGP) and CHH. L173R of *PROKR2* accounts for 40% of the CHH population in Europe and the United States; W178S of *PROKR2* accounts for 58.8% of Chinese CHH patients. Micropenis and cryptorchidism are important cues for CHH in children. They are more common in pediatric patients than in adult patients. It is not rare of Leydig cell dysfunction (dual CHH), neither of oligogenic mutations diagnosed CHH in children. Both *PROKR2* and *KISS1R* maybe the potential shared pathogenic genes of CDGP and CHH, and W178S in *PROKR2* may be a founder mutation in Chinese CHH patients.

**Keywords: congenital hypogonadotropic hypogonadism, clinical and genetic characteristics, oligogenicity, dual CHH, family history**

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## INTRODUCTION

Congenital hypogonadotropic hypogonadism (CHH, MIM 615267) is a common cause of absent puberty and adult infertility, with an incidence rate of 1 per 4000 new births (1). When associated with anosmia or hyposmia, it is also known as Kallmann syndrome (KS, MIM 147950). When associated with a normal sense of smell, it is termed normosmic CHH (nHH), which accounts for 50% of the cases (2). There are approximately 1200–1500 gonadotropin-releasing hormone (GnRH) neurons in the vertebrate hypothalamus that can synthesize and release GnRH. CHH is caused by a deficiency in the synthesis, release, or action of GnRH, resulting in insufficient secretion of gonadotropins, followed by gonadal dysfunction.

Congenital male hypogonadism is divided into three types according to clinical manifestations and hormone levels: central (hypothalamic-pituitary), primary (testicular), and combined forms (hypothalamic-pituitary and testicular, the so-called dual hypogonadism) (3, 4). As a disease paradigm of GnRH deficiency, CHH provides insight into the physiology and pathophysiology of the hypothalamic-pituitary-gonadal axis (HPG). A follow-up study of adult CHH patients treated with GnRH pumps reported that 11.1% (10/90) of the patients remained hypogonadotropic and hypogonadal. This suggests that the patients had both pituitary and testicular defects. Only 8.9% (8/90) of patients achieved spermatogenesis and normal T, but with hypergonadism, demonstrating poor testicular responsiveness to gonadotropins. Also, 5.6% (5/90) of patients had azoospermia but with adult testicular volumes and normal hormonal levels, indicating primary defects in spermatogenesis, which is referred to as dual CHH (5).

According to its pathophysiology, CHH is mainly divided into two types. During the fetal period, neurodevelopmental gene mutations cause disorders in the development, differentiation, or migration of GnRH neurons, usually causing KS. Defects in GnRH synthesis, release, or action in pituitary gonadotropin cells caused by neuroendocrine gene mutations usually lead to nHH (1). Many studies have found that CHH can be caused by gene defects that affect both neuronal development and the GnRH signaling pathway. Mutations in the same CHH-related pathogenic gene often cause phenotypic differences among patients or individuals in the same family; the low penetrance of most genes suggests that CHH is not a strictly monogenic disease (6, 7). Studies including large CHH cohorts have suggested that at least 20% of CHH cases are oligogenic (7, 8). However, our previous study involving 64 patients indicated that oligogenic mutations accounted for only 9.8% of the mutations (9).

Since the first KS-related pathogenic gene, *ANOS1* was cloned in 1991, many CHH-related pathogenic genes have been identified. In 2015, the European CHH consensus summarized 31 pathogenic genes, including X-chromosome-linked recessive, autosomal recessive, and dominant genes (1). At present, more than 90 candidate genes may be involved in the pathogenesis of CHH, and some newly reported genes have been confirmed in CHH patients. However, some genes involved in GnRH neuronal migration and axon formation in animal models have not been confirmed in CHH patients (8, 10–16). In our previous study, only 10 pathogenic genes were identified in patients with CHH (9).

Men with CHH exhibited decreased trabecular thickness and lower cortical bone area despite long-term hormonal treatment. Early treatment during adolescence may enhance trabecular outcomes, highlighting the importance of early diagnosis and interval (17). Therefore, this study aimed to evaluate the relationship between genotypes and phenotypes in pediatric CHH, thus providing more evidence for early diagnosis and intervention.

## MATERIALS AND METHODS

### Ethical Considerations

The study was approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University, and written informed consent was obtained from the parents or legal guardians of the patients. All necessary data involved in the study were available.

### Subjects

A total of 125 male patients of Chinese Han nationality aged 0–18 years, who were treated at the endocrine clinic of our hospital between 2008 and 2020, were enrolled. The patients were not related to each other.

The diagnosis was made based on clinical expression and laboratory investigations, including sex hormone levels (AMH and INHB), olfactory bulb magnetic resonance imaging (MRI), hCG test, chromosome karyotype, and genetic analysis. The testicular volume in all patients was evaluated using a Prader Willy orchydometer.

### Inclusion Criteria

The inclusion criteria were as follows: (1) No puberty initiation (testicular volume < 4 ml) with or without genitourinary malformation (micropenis, cryptorchidism and hypospadias), and baseline serum testosterone ≤ 20 ng/dl and gonadotrophins (LH, FSH) at a prepubertal levels, or with puberty stagnation. The LHRH stimulation test results could be used as a reference when bone age >12 years; (2) There are no occupying lesions of pituitary and hypothalamus on MRI; (3) KS or nHH is depended on questionnaire of anosmia/hyposmia, and MRI of olfactory bulb; (4) Follow-up was needed to rule out delay growth and puberty and isolated growth hormone deficiency (GHD); (5) Molecular genetic testing supported the diagnosis (All mutations were predicted to be pathogenic, likely pathogenic or uncertain).

### Exclusion Criteria

The exclusion criteria were as follows: (i) Any ascertained diseases (for example, chromosomal abnormality, trauma, surgeries, congenital adrenal hyperplasia (CAH), NR5A1-related disorders of sex development) or other ascertained diseases accompanied by sex agenesis (such as Prader-Willi syndrome (PWS), multiple pituitary hormone deficiency); (ii) Presence of chronic systemic diseases (for example,

uraemia, thalassaemia, poorly controlled diabetes); (iii) Protein-energy malnutrition; (iv) Eating disorders (for example, anorexia nervosa, bulimia); (v) Intracranial lesions or pituitary tumors.

## The Diagnostic Criteria of Micropenis

Micropenis was diagnosed according to the criteria of the Chinese Journal of Pediatric Surgery in 2010 (18).

## Diagnostic Criteria of Dual CHH

After the hCG prolongation test, the testosterone (T) level was still less than 100 ng/dL and the patient was diagnosed with dual CHH. If the hCG prolongation test was not performed, the level of T was less than 100 ng/dL after treatment with GnRH (5–10 ug/90 min, 16 pulses/d) for half a year, and the patient was diagnosed with dual CHH. If the hCG prolongation test was not performed, GnRH treatment was provided for less than half a year, and the level of T was still greater than 100 ng/dL, testicular Leydig cells were considered to have a good response.

## hCG Standard and Prolongation Tests

HCG standard test and prolongation test were performed as previously described by Wang et al. (9).

## Hormone Detection

LH, FSH, and T levels were measured using an enzyme-enhanced chemiluminescence immunoassay (Immulite 2000; Siemens Corporation, Munich, Germany). Normal laboratory levels for T is 180-608ng/dl. INHB was measured using Chemiluminescence immunoassay (iflash 3000-c chemiluminescence immunoanalyzer, Shenzhen yahuilong Biotechnology Co., Ltd, Shenzhen, China), normal laboratory levels for INHB is 18.22-311.27pg/ml.

## DNA Sequence Analysis

A total of 51 patients underwent gonadal panel analysis, including 164 genes, and 44 patients underwent whole-exome sequencing. All 164 genes were screened by referencing the OMIM and HGMD databases. We input the keywords ("idiopathic GnRH deficiency", "congenital hypogonadotropic hypogonadism", "complex hypogonadism", "Kallmann syndrome", "gonad dysgenesis", "micropenis", "cryptorchidism", "hypospadias" and "disorders of sex development") in Pubmed. The genes included in targeted next-generation sequencing (NGS) are listed in **Supplementary Table 1**. DNA was extracted from the peripheral blood leukocytes of patients and their parents and/or siblings. A NEXTSEQ 500 sequencer (Illumina Corporation, San Diego, CA, USA) was then used to filter out all possible pathogenic missense, frameshift, and splice site mutations. Design primers and Sanger sequencing were used to verify mutations in the samples. Missense mutations were assessed according to the American College of Medical Genetics and Genomics (ACMG) rules (19), and both frameshift and splicing sites were considered pathogenic mutations.

For whole-exome sequencing (The work was performed in Beijing Mygenostics co., LTD, Beijing, China), process is as follows: 1)DNA Library Preparation: Approximately 2 mL peripheral blood (EDTA anticoagulant) of the patient was collected, and genomic DNA was extracted using QIAamp Blood Midi Kit (QIAGEN, Germany) according to the instructions. Paired-end sequencing libraries then were prepared using a DNA sampleprep reagent set 1 (NEBNext). Library preparation included end repair, adapter ligation and PCR enrichment, and was carried out as recommended by Illumina protocols; 2) Targeted genes enrichment and sequencing: The amplified DNA was captured use GenCap Whole-exome capture kit (MyGenostics GenCap Enrichment technologies). The capture experiment was conducted according to manufacturer's protocol. The average sequencing depth > 100X, fraction of target covered with at least 10X > 95%. The enrichment libraries were sequenced on Illumina HiSeq X ten sequencer for paired read 150bp; 3)Bioinformatics analysis: After sequencing, the rawdata were saved as a FASTQ format, then followed the bioinformatics analysis: First, Illumina sequencing adapters and low quality reads (<80bp) were filtered by cutadapt. After quality control, the clean reads were mapped to the UCSC hg19 human reference genome using BWA (http://bio-bwa.sourceforge.net/). Duplicated reads were removed using picard tools and mapping reads were used for variation detection. Second, the variants of SNP and InDel were detected by GATK HaplotypeCaller, then using GATK VariantFiltration to filter variant. After above two steps, the data would be transformed to VCF format, variants were further annotated by ANNOVAR and associated with multiple databases, such as,1000 genome, ESP6500, dbSNP, EXAC, Inhouse (MyGenostics), HGMD, and predicted by SIFT, PolyPhen-2, MutationTaster, GERP++; 4) Variants Selected: In this course, five steps using to select the potential pathogenic mutations in downstream analysis: (i) Mutation reads should be more than 5, mutation ration should be no less than 30%; (ii) Removing the mutation, the frequency of which showed more than 5% in 1000g, ESP6500 and Inhouse database; (iii) If the mutations existed in InNormal database (MyGenostics), then dropped; (iv) Removing the synonymous. (v) After (i), (ii), (iii), if the mutations were synonymous and they were reported in HGMD, left them. When finished above jobs, the mutations which were left should be the pathogenic mutations. All mutations of minor allele frequency < 1% in East Asian people, and pathogenic, likely pathogenic, or uncertain mutations were possibly related to the disease.

## Statistical Analysis

Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA). When comparing the two groups, independent sample t-test is used for normal distribution, and Mann-Whitney U-test is used for non-normal distribution, Chi square test was used to compare the proportion of cryptorchidism between KS and nHH groups. It is considered to be statistically significance when $p < 0.05$.

# RESULTS

## Clinical Characteristics

The chromosomes of all the patients were 46 XY and SRY (+). Combined with the phenotypes, physical signs, hormone levels, presence of puberty, olfactory bulb imaging, and genetic test

results, a total of 125 cases of CHH were diagnosed, including 87 cases of KS (69.6%), 37 cases of nHH (29.6%), and 1 case of CHARGE syndrome. Only 5 patients (4%) were diagnosed 6 months after birth (**Figure 1A** and **Supplementary Table 2**). The results of 64 patients have been published elsewhere (9).

In total, 80.0% of the patients (100/125) had micropenis, including those with micropenis (38.4%, 48/125), micropenis with cryptorchidism (35.2%, 44/125), micropenis with cryptorchidism and hypospadias (2.4%, 3/125), micropenis with hypospadias (3.2%, 4/125), cryptorchidism (8.8%, 11/125), and absent puberty (12.0%, 15/125) (**Figure 1B** and **Table 1**).

We further compared the clinical characteristics of KS and nHH and found that the incidence of micropenis in KS patients was higher. However, there were no significant differences in the incidence of cryptorchidism, testicular volume, length of micropenis, testosterone levels after hCG standard and prolongation tests (**Table 2**).

In total, 2 patients with KS had left renal agenesis (one with *an ANOS1* mutation and the other without gene detection). In 1 patient with *an FGFR1* mutation, bimanual synkinesis was observed. A total of 65 patients with KS completed an MRI examination of the olfactory bulb, and 7.7% (5/65) reported hyposmia. However, no abnormal olfactory bulb, olfactory tract, or olfactory sulcus was found on the MRI. Based on the description and observation of olfaction by children and their parents, normal olfactory function was reported in 30.8% of the cases (20/65). Meanwhile, structural abnormalities of the olfactory bulb, olfactory bundle, and/or olfactory sulcus were

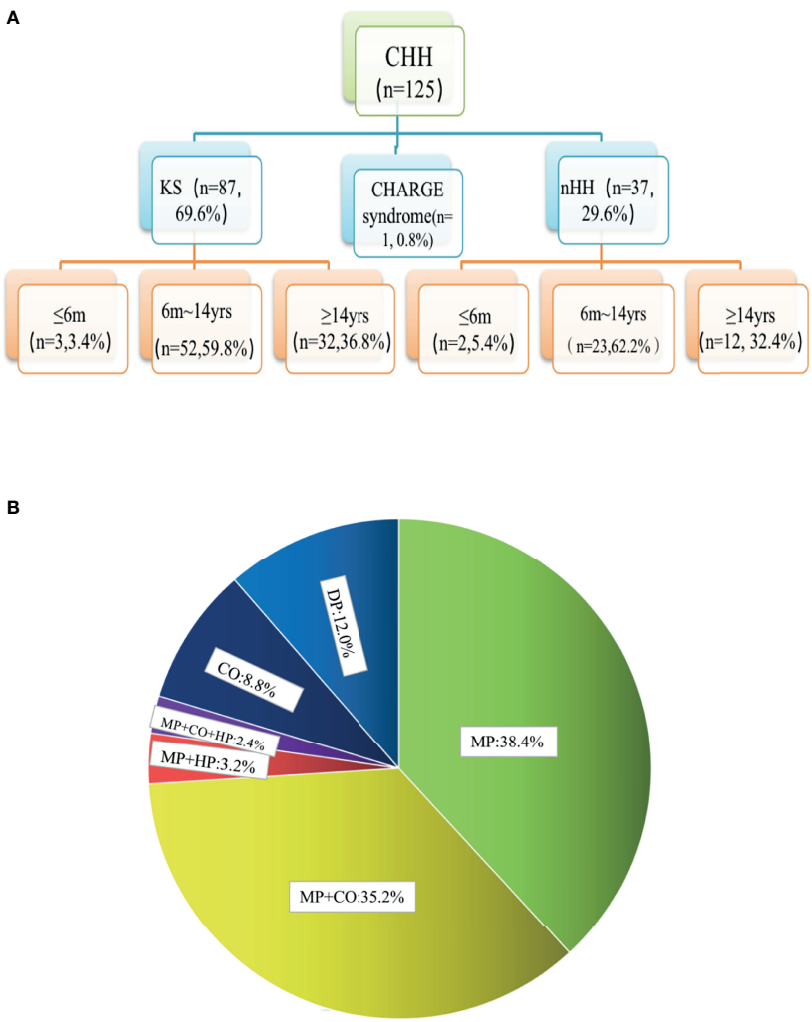


**FIGURE 1** | Age distribution and phenotypic proportion of 125 patients with CHH. **(A)** In our group of patients with KS and nHH, more than half were diagnosed before the age of 14, one third were diagnosed over the age of 14, and a few cases could be diagnosed within 6 months due to the lack of puberty. **(B)** 80.0% of the patients had micropenis, including those with micropenis (38.4%), micropenis with cryptorchidism (35.2%), micropenis with cryptorchidism and hypospadias (2.4%), micropenis with hypospadias (3.2%), 8.8% of the patients had cryptorchidism and 12.0% had absent puberty. MP, micropenis, CO, cryptorchidism; HP, hypospadias; DP, delayed puberty.

**TABLE 1** | Genetic results of 7 patients with hypospadias.

| No. | Date of birth | Diagnosis | Age* (yrs) | First gene | Nucleotide | Amino acid | Second gene | Nucleotide | Amino acid | Third gene | Nucleotide | Amino acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2012/3/15 | KS | 0.42 | HS6ST1 | c.187C>G | p.H63D | SALL1 | c.1984A>G | p.M662V | | | |
| 2 | 2003/7/3 | KS | 3.42 | ANOS1 | c.958G>A | p.E320K | | | | | | |
| 3 | 2000/9/11 | KS | 9 | FGF17 | c.359C>T | p.P120L | | | | | | |
| 4 | 2005/8/30 | KS | 10.17 | CHD7 | c.3247A>G | p.T1083A | CHD7 | c.6379G>A | p.A2127T | HS6ST1 | c.1177G>A | p.D393N |
| 5 | 2003/9/26 | KS | 12 | ANOS1 | c.1678G>A | p.V560I | | | | | | |
| 6 | 2017/5/6 | nHH | 3.25 | PROKR2 | c.472G>A | p.V158I | SPECC1L | c.694A>G | p.M232V | | | |
| 7 | 2009/6/4 | nHH | 14 | Negative | | | | | | | | |

**Age at diagnosis.*

**TABLE 2** | Baseline clinical characteristics of 125 pediatric patients with CHH.

| Items | KS (n = 87) | nHH (n = 38) | p value |
|---|---|---|---|
| Age at evaluation (yr) | 13.0 (11.0, 14.8) | 10.3 (3.9, 14.4) | 0.08 |
| Puberty (%) | | | |
| Partial | 1.1 | 7.9 | 0.156 |
| No | 98.9 | 92.1 | |
| Cryptorchidism (%) | | | |
| Unilateral | 27.6 | 21.1 | 0.451* |
| Bilateral | 21.8 | 21.1 | |
| No | 50.6 | 57.9 | |
| Microphallus (%) | | | |
| Yes | 86.2 | 65.8 | 0.009 |
| Length of penis (cm) | 3.1 (2.5, 4.0) | 3.7 (2.5,4.9) | 0.124 |
| Testicular volume (ml) | 1.5 (1.0, 2.0) | 2.0 (1.0, 2.5) | 0.534 |
| T after hCG standard test (ng/dl) | 44.2 (20.3, 115.2) | 42.0 (20.0 156.5) | 0.994 |
| T after hCG prolonged test (ng/dl) | 118.0 (64.7, 189.3) | 138.4 (50.1, 185.3) | 0.913 |

**Denotes the comparison of the proportion of cryptorchidism between KS and nHH groups. It is considered that there is a statistical difference When P < 0.05.*

observed on MRI, consistent with the results found in the literature and our previous reports. The MRI images of 3 patients with KS are shown in **Figure 2**. Patients could show structural abnormalities, for example, unilateral or bilateral olfactory bulb and olfactory tract cannot be displayed and shallow olfactory sulcus. However, on the other hand, some patients claim that they have normal smell, but the MRI examination is not perfected. These patients cannot be excluded the diagnosis of KS. Therefore, the proportion of KS in this study may be higher.

## Genetic Characteristics

The karyotype of all patients was 46; XY and the SRY gene was normal for NGS detection. A total of 94 patients underwent genetic testing: 25 CHH-related pathogenic genes were found in 81.9% of cases (77/94), 15 new CHH-related genes were confirmed in our patients compared to previous studies (**Figure 3A**), digenic mutations in 24.5% of cases (23/94), and trigenic mutations in 3.2% of cases (3/94) (**Table 3** and **Figures 3B, C**).

The most common mutations were *FGFR1* (20/94, 21.3%), *PROKR2* (17/94, 18.1%), *CHD7* (12/94, 12.8%), and *ANOS1* (11/94, 11.7%). Among the most common gene variants, there was no significant difference in the proportion of *FGFR1*, *PROKR2*, and *CHD7* variants between KS and nHH (all p>0.05).

Most mutations in CHH probands were private, except for several sites, such as W178S of *PROKR2* (n=5 in KS and nHH, respectively), V560I of *ANOS1* (n=2 in KS), H63D of *HS6ST1* (n=3 in KS), and P191L and S671L of *IL17RD* (n=2 in KS, respectively) (**Figure 3A**).

The oligogenicity of common autosomal dominant inherited pathogenic genes accounted for 50% (*FGFR1*, 10/20) and 33.3% (*CHD7*, 4/12). The oligogenicity of autosomal recessive inherited pathogenic genes accounted for 47.1% (*PROKR2*, 8/17, **Figure 3D**), and the oligogenicity of X-linked genes accounted for 9.1% (*ANOS1*, 1/11). Among the 12 patients with *CHD7* variants, only 1 patient (8.3%) was diagnosed with CHARGE syndrome. Among the 11 patients with KS caused by the *ANOS1* variant, 1 patient had exon 1 and 2 deletions, and another had all exon deletions.

We further analyzed the common pathogenic gene mutation sites according to ACMG. We found that 45.5%–75.0% of the mutation sites were pathogenic or likely pathogenic, except for 1 case of the *CHD7* variant being likely benign. The rest of these patients had either single-gene or oligogenic mutations (**Figure 3E**).

## Dual CHH and Genes

In total, 105 of 125 patients completed the hCG standard test to evaluate testicular Leydig cell function. Of these 105 patients, 68.6% (72/105) had T<100 ng/dL, of whom 65 patients completed the hCG prolongation test, and 22.9% (24/105) of patients had T<100 ng/dL, suggesting testicular Leydig cell dysfunction. Therefore, at least 22.9% of the cases could be diagnosed as having dual CHH (**Figure 4**).

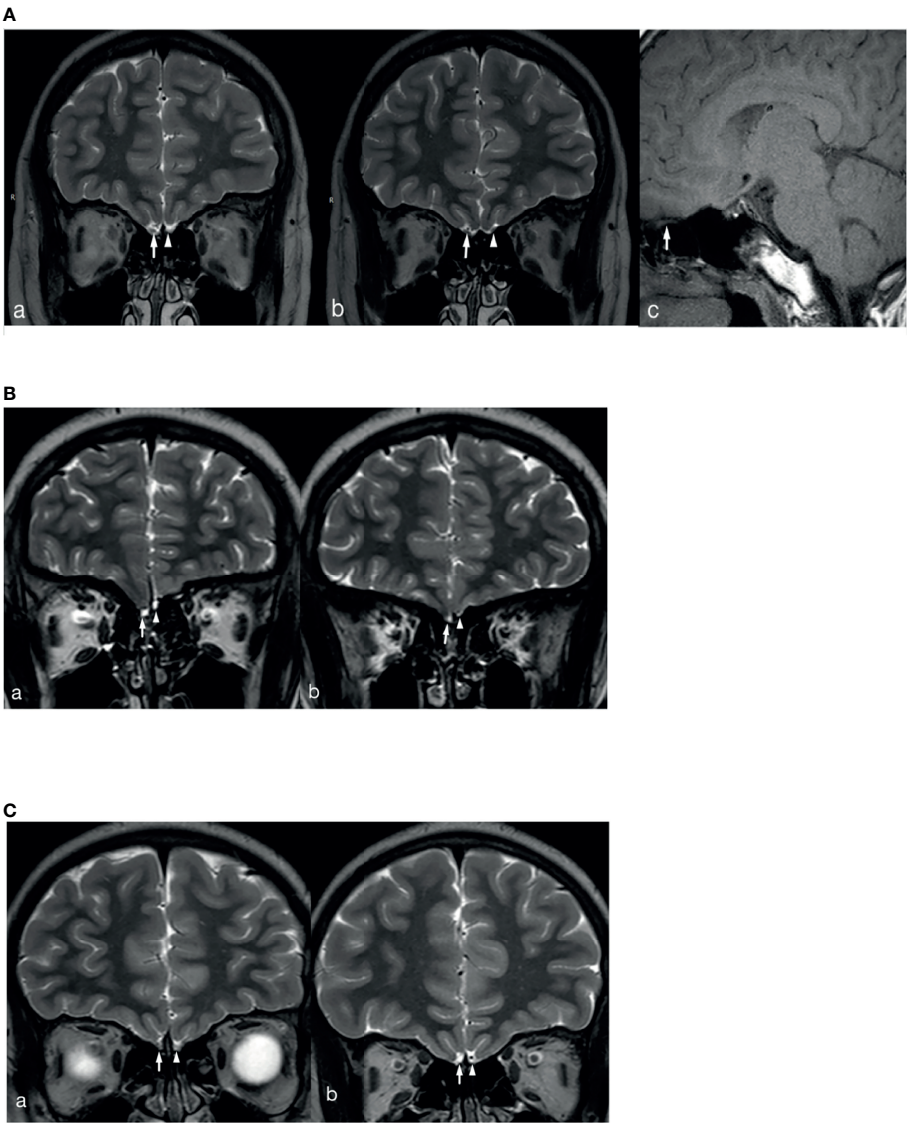


**FIGURE 2** | MRI images of 3 patients with KS. **(A)** The right olfactory bulb and olfactory tract of the patient are normally displayed (White long arrows in a and b show the right olfactory bulb and olfactory tract, white long arrow c shows sagittal position of right olfactory tract), the left olfactory bulb is not displayed (White short arrow in a), and the left olfactory tract (White short arrow in b) is smaller than the opposite side. **(B)** The bilateral olfactory bulbs and olfactory tracts of the patient are not clearly displayed (White long arrows in a and b show the right olfactory bulb and olfactory tract, white short arrows in a and b show the left olfactory bulb and olfactory tract), and the left olfactory sulcus is shallower than the opposite side (a and b). **(C)** The bilateral olfactory bulbs and olfactory tracts of the patient are not clearly displayed (White long arrows in a and b show the right olfactory bulb and olfactory tract, white short arrows in a and b show the left olfactory bulb and olfactory tract), and the bilateral olfactory sulcus is shallow (a and b).

## Family History and Genes

Among 39 patients with positive family histories, 56.4% (22/39) had a family history of delayed puberty (DP). In addition to the CHH-related genes (*FGFR1*, *HS6ST1*, *IL17RD*, and *SEMA3A*) reported in the literature for patients with DP (20, 21), 2 patients had *PROKR2* mutations in late-developing mothers (menarche at 16 years), and 1 carried *KISS1R* mutations in a late-developing father (first spermatorrhea at the age of 18–19 years), suggesting that *PROKR2* and *KISS1R* may also be shared genes of DP and CHH (**Figure 5**).

Seven families had CHH (**Figure 6**). In family 1, there were 3 KS brothers with *ANOS1* deletion of exons 1 and 2, and the eldest brother began to receive GnRH pump therapy in another adult hospital. In family 2, both KS brothers carried *ANOS1* missense mutation (C164S). In family 3, both KS brothers carried another *ANOS1* missense mutation (V560I). In family 4, there was 1 nHH patient with *K1SS1R* compound heterozygous mutations, and his elder sister carried the same mutation without puberty signs at the age of 17 years. In families 5-7, the three mothers were probands, and they were all treated with estrogen and

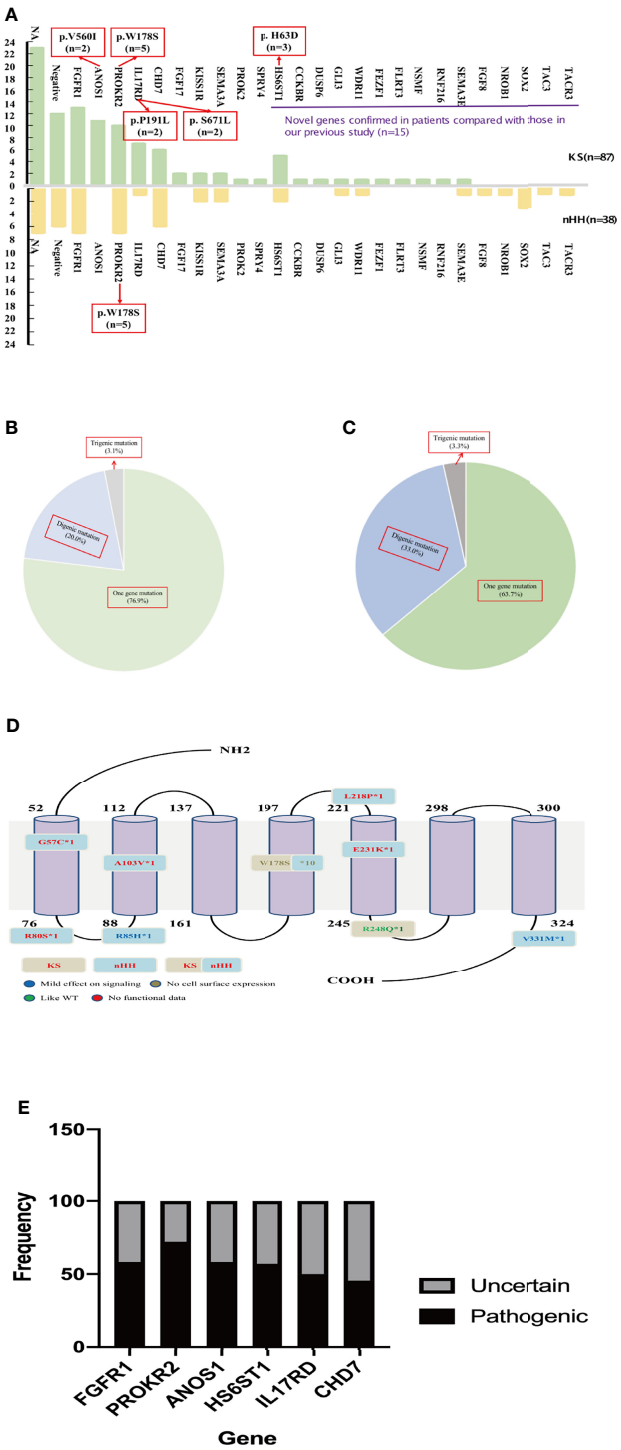


**FIGURE 3** | Gene analysis of 125 patients. **(A)** The gene distribution of patients with KS and nHH. As can be seen from the **Figure 2A**, 15 new genes were confirmed in patients compared with our previous report. Multiple genes (*ANOS1*, *PROKR2*, *IL17RD* and *HS6ST1*) were found to have repeated mutation sites. The gene of the patient with CHARGE syndrome was classified as nHH. **(B)** Digenic and trigenic gene mutations accounted for nearly a quarter of KS patients; **(C)** Digenic and trigenic gene mutations accounted for more than one third of nHH patients. **(D)** *PROKR2* was one of the most common genes in CHH patients, 18 mutation sites were found in 17 patients, mostly loctated in transmembrane region and intracellular segment, and W178S accounted for 58.8% (10/17 patients), which may could be as the founder mutation of Chinese CHH patients. **(E)** We further analyzed the ratio of common gene pathogenic/possible pathogenic mutations to uncertain mutation sites in this study, and found that 45.5% - 75.0% of the mutation sites were pathogenic or like pathogenic. For patients with uncertain mutation sites, we need longer follow-up to confirm its diagnosis and mutation pathogenicity.

**TABLE 3 |** Oligogenic mutations of 26 patients.

| Case | Diagnosis | Number of gene | Number of Mutant Alleles | Gene | Nucleotide Change | Amino Acid Change | Mutation Type |
|---|---|---|---|---|---|---|---|
| 1 | KS | 3 | 3 | SEMA3E | c.760G>C | p.E254Q | missense |
| | | | | CHD7 | c.2824A>G | p.T942A | missense |
| | | | | NSMF | c.188C>T | p.P63L | missense |
| 2 | KS | 3 | 3 | FGFR1 | c.340-344delTTCTC | p.F114fs13 | frameshift |
| | | | | FEZF1 | c.614C>T | p.A205V | missense |
| | | | | FLRT3 | m.2556G>A | | noncoding region |
| 3 | nHH | 3 | 4 | PROKR2 | c.533G>C | p.W178S | missense |
| | | | | CHD7 | c.*480_*481insAGGC | | UTR |
| | | | | CHD7 | c.*480_*481insCAGTATGCT CGGGACGCCCTGGCTAAGAA CATCTACAGCCGCC | | UTR |
| | | | | FGF8 | c.-72A>G | | UTR |
| 4 | KS | 2 | 3 | FGFR1 | c.801C>G | p.Y267Ter | missense |
| | | | | PROKR2 | c.743G>A | p.R248Q | missense |
| | | | | PROKR2 | c.533G>C | p.W178S | missense |
| 5 | KS | 2 | 2 | FGFR1 | c.1034_c.1035del | p.S345Cfs54* | frameshift |
| | | | | ANOS1 | c.907G>A | p.V303I | missense |
| 6 | KS | 2 | 2 | FGFR1 | c.736C>T | p.R246W | missense |
| | | | | CHD7 | c.8250T>G | p.F2750L | missense |
| 7 | KS | 2 | 2 | FGFR1 | c.1704+1G>A | | splicing site |
| | | | | SPRY4 | c.88C>T | p.R30W | missense |
| 8 | KS | 2 | 2 | PROKR2 | c.533G>C | p.W178S | missense |
| | | | | PROK2 | c.301C>T | p.R101W | missense |
| 9 | KS | 2 | 2 | PROKR2 | c.691G>A | p.E231K | missense |
| | | | | IL17RD | c.192A>G | p.M658V | missense |
| 10 | KS | 2 | 3 | PROKR2 | c.239G>A | p.R80H | missense |
| | | | | PROKR2 | c.169G>T | p.G57C | missense |
| | | | | SEMA3A | c.1453-9delG | | frameshift |
| 11 | KS | 2 | 2 | IL17RD | c.1319G>T | p.G440V | missense |
| | | | | GLI3 | c.1930G>A | p.G644R | missense |
| 12 | KS | 2 | 3 | IL17RD | c.572C>T | p.P191L | missense |
| | | | | ANOS1 | c.1654G>A | p.E552K | missense |
| | | | | ANOS1 | c.1062+1G>A | - | splicing site |
| 13 | KS | 2 | 2 | KISS1R | c.149C>A | p.A50E | missense |
| | | | | CCKBR | c.1247G>A | p.R416H | missense |
| 14 | KS | 2 | 3 | CHD7 | c.3247A>G | p.T1083A | missense |
| | | | | CHD7 | c.6379G>A | p.A2127T | missense |
| | | | | HS6ST1 | c.1177G>A | p.D393N | missense |
| 15 | KS | 2 | 2 | FGF17 | c.580C>G | p.Q194E | missense |
| | | | | CHD7 | c.7912A>G | p.I2638V | missense |
| 16 | KS | 2 | 2 | FGFR1 | c.1439T>G | p.L480X | truncation |
| | | | | SEMA3A | c.1306G>A | p.V436I | missense |
| 17 | nHH | 2 | 2 | FGFR1 | c.238C>T | p.R80C | missense |
| | | | | SOX2 | c.695C>A | p.T232N | missense |
| 18 | nHH | 2 | 2 | FGFR1 | c.142G>A | p.G48S | missense |
| | | | | GLI3 | c.3286G>A | p.V1096M | missense |
| 19 | nHH | 2 | 2 | PROKR2 | c.308C>T | p.A103V | missense |
| | | | | SEMA3E | c.760G>C | p.E254Q | missense |
| 20 | nHH | 2 | 2 | PROKR2 | c.533G>C | p.W178S | missense |
| | | | | CHD7 | c.6955C>T | p.R2319C | missense |
| 21 | nHH | 2 | 2 | SOX2 | c.330C>A | p.Y110Ter | missense |
| | | | | SEMA3A | c.1432G>A | p.E478K | missense |
| 22 | nHH | 2 | 2 | SOX2 | c.6955C>A | p.T232N | missense |
| | | | | CHD7 | c.2656C>T | p.R886W | missense |
| 23 | nHH | 2 | 2 | HS6ST1 | c.1177G>A | p.D393N | missense |
| | | | | TAC3 | c.107G>A | p.R36H | missense |
| 24 | nHH | 2 | 2 | KISS1R | c.929G>A | p.C310Y | missense |
| | | | | NROB1 | c.379G>A | p.A127T | missense |
| 25 | nHH | 2 | 3 | FGFR1 | c.-55A>G | | noncoding region |

*(Continued)*

**TABLE 3** | Continued

| Case | Diagnosis | Number of gene | Number of Mutant Alleles | Gene | Nucleotide Change | Amino Acid Change | Mutation Type |
|---|---|---|---|---|---|---|---|
| | | | | FGFR1 | c.1825-30G>A | | intron |
| | | | | PROKR2 | c.533G>C | p.W178Se | missense |
| 26 | nHH | 2 | 2 | WDR11 | c.386G>A | p.R129H | missense |
| | | | | SEMA3A | c.2200C>T | p.R734W | missense |

*Only 5 patients carried digenic mutations in our previous study, while in this study, the proportion of digenic and trigenic mutations was significantly higher than that before (27.7% vs. 9.8%).*

**Means mutation leads to amino acid termination.*

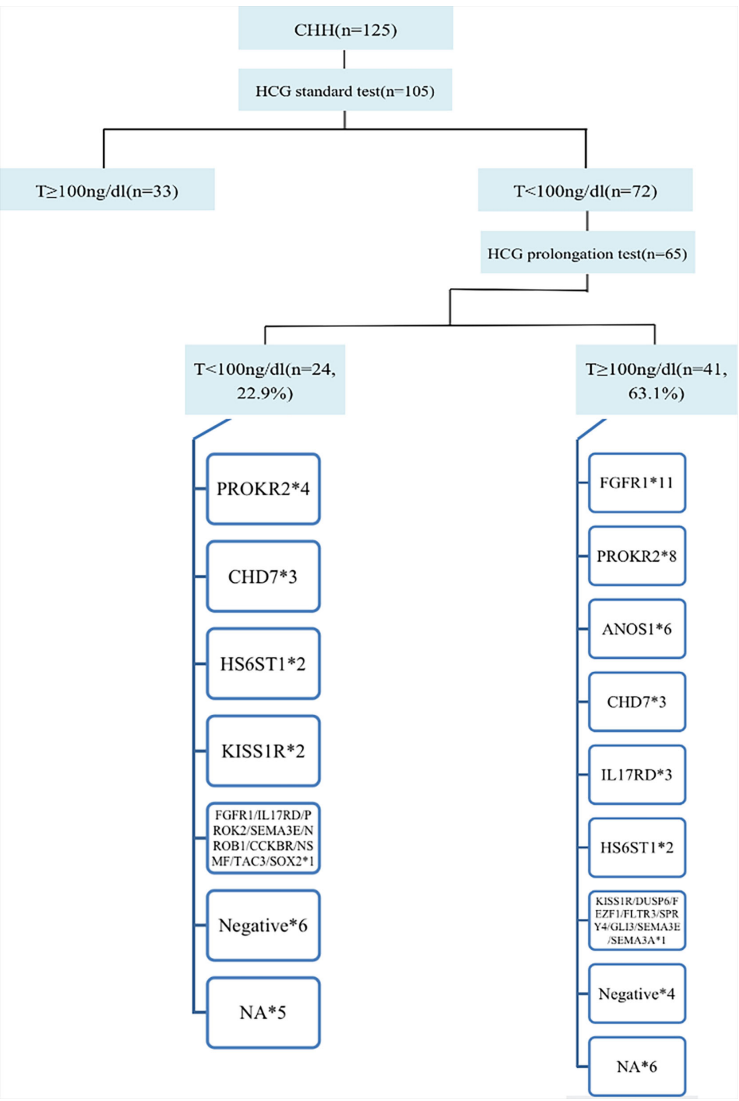


**FIGURE 4** | Diagnosis process and gene distribution of dual CHH. 65 patients continued to perform the hCG prolongation test, and T levels of 22.9% (24/105) patients were lower than 100ng/dl, suggesting that these patients also had testicular Leydig cell dysfunction (Dual CHH). In patients with dual and pure CHH, ratio of *FGFR1* mutation was 5.3% (1/19) vs. 31.4% (11/35), and there was no significant difference between the two groups (P = 0.138). *represents the number of cases with the same gene mutation.

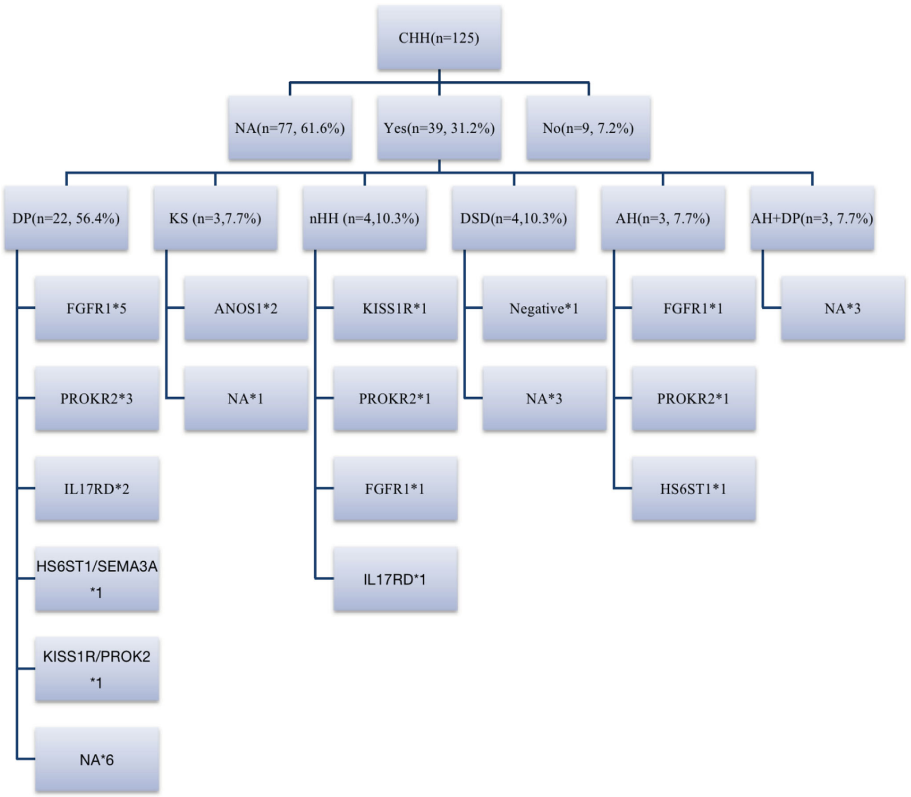


**FIGURE 5** | Family history and genes of patients with CHH. 31.2% (39/125) of patients could be traced back to positive family history, including DP, CHH, DSD, abnormal smell, abnormal smell combined with DP family history. 18% (7/39) of patients had KS/nHH family history, and more than half of patients had DP family history, *FGFR1*, *IL17RD*, *HS6ST1* and *SEMA3A* are shared genes of CHH and DP reported before, while in our cohort, we believe that *PROK2/PROKR2* and *KISS1R* are also potential shared genes. DP, delayed puberty; DSD, disorders of sex development; AH, anosmia or hyposmia. *represents the number of cases with the same gene mutation.

progesterone to regulate their menstrual cycles. When they had fertility needs, they were administered GnRH pump therapy combined with assisted reproductive technology and carried three gene mutations (N503S of *IL17RD*, P176S of *FGFR1*, and W178S of *PROKR2)*.

## DISCUSSION

This study summarized the clinical and genetic characteristics of 125 patients with CHH using CHH and DSD phenotypes (micropenis and cryptorchidism) and genetic testing as important cues for pediatric diagnosis of CHH. With an increase in the number of cases, more CHH candidate genes have been confirmed in patients. We found that digenic and trigenic variants accounted for 24.5% (23/94) and 3.2% (3/94) of the patients, respectively.

The most common mutant genes were similar to those reported in the literature, but mutations in each gene identified accounted for <10% of cases in previous studies (7, 8, 22–24), The proportion of common genes was significantly higher in our study: *FGFR1* (21.3%), *PROKR2* (18.1%), *CHD7* (12.8%), and *ANOS1* (11.7%). Among them, 10 cases (50.0%) of *FGFR1* and 7 cases (41.2%) of *PROKR2* mutations were oligogenic mutations. Previous studies have reported that *FGFR1* mutation may be related to hand and foot malformations in patients with CHH (22). However, in this study, only 1 patient with hand and foot malformations had *FGFR1* mutation; there was no obvious phenotypic-genotypic correlation, probably because more than half of the patients were oligogenic.

Previous studies on HH in adults reported that patients with *FGFR1* mutations had a high incidence of cryptorchidism, small testicular size, long treatment time for spermatogenesis, and low sperm concentration (25). However, in this study, compared with patients with non-*FGFR1* mutations, the incidence of cryptorchidism in patients with *FGFR1* mutations was not significantly higher, the testicular volume was not small, and the patients with *FGFR1* mutations were mainly pure CHH. However, further follow-up studies should be conducted because the diagnosis of pure CHH in this study was based on the T level after the hCG prolongation test, which may explain the difference.

INHB is a marker of the number of Sertoli cells and is usually lower than 30 pg/mL in male patients with complete CHH

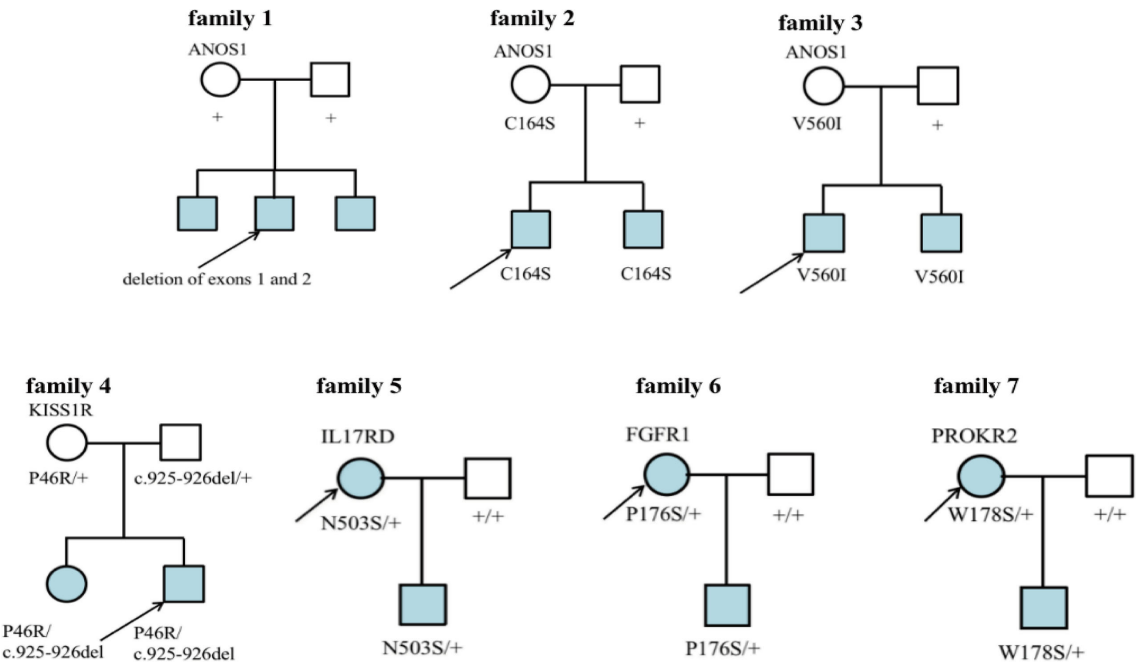


**FIGURE 6** | Family map of 7 CHH patients. Families 1-3 were KS patients, all probands harbored ANOS1 mutation. Families 4-7 were nHH patients. In families 5-7, mothers of the patiens were probands and gave birth to children after treatment, which further confirmed that N503S of *IL17RD*, P176S of *FGFR1* and W178S of *PROKR2* had relatively little effect on reproduction and could restore reproductive function after treatment. + denotes wild-type allele.

(1, 26). In some patients with partial CHH, the levels of INHB may overlap with those of DP and healthy controls (27, 28). In this study, the INHB test was performed in 65 patients, including all age groups, of which 45 (69.2%) had an INHB level of >30 pg/mL, suggesting that the function of testicular Sertoli cells in these patients was still good. Subsequent therapy with GnRH pump or hCG/HMG was more likely to promote spermatogenesis, consistent with the current spermatogenesis rate of 64.0%–80.3% in CHH patients after treatment (29, 30). Among the 39 patients with T>100 ng/dL after the hCG prolongation test, there were still 10 patients whose INHB level was <30 pg/mL, suggesting that some patients with a good response to Leydig cells may still have poor function of Sertoli cells. Therefore, evaluation of testicular cell function in patients with HH requires a multi-faceted and multi-index comprehensive evaluation.

However, in this study, 1 patient with three gene mutations (*SEMA3E/CHD7/NSMF*) was diagnosed with dual CHH. The patient was treated with a standard GnRH pump for 6 months, and the level of T was 112 ng/dL. After 12 months, it was observed that the patient had spermatorrhea and good sperm motility and concentration. Therefore, the percentage of patients diagnosed with dual CHH in this study with restored fertility after treatment requires further study. This phenomenon also suggests that there was a false-negative response to the short-term stimulation in the experiment.

In previous studies on adult cases, patients whose puberty was not induced by hormone therapy usually showed infertility when GnRH pump therapy was used to stimulate spermatogenic potential in adults. Therefore, for patients with CHH and their families, every gene mutation that causes GnRH deficiency should theoretically not be transmitted within the family. Recent studies have shown that a small number of gene mutation sites have strikingly high percentages; for example, the percentages of Q106R and R262Q in *GNRHR* were 44% and 29%, respectively (31–33) and W275X of *TACR3* was 36% (34–36). The L173R of *PROKR2* accounts for 40% of the CHH population in Europe and the United States but is rare in Asian populations (37–40). The recurrent mutation sites of several genes in this study were W178S of *PROKR2* (n=5 in KS and nHH), V560I of *ANOS1* (n=2 in KS), H63D of *HS6ST1* (n=3 in KS), and P191L and S671L of *IL17RD* (n=2 in KS). It was proven that *PROKR2* was one of the most common pathogenic genes in CHH, accounting for 17.9% (17/95) of genes in this study, W178S accounted for 58.8% (10/17). In another study of Chinese adult CHH patients, *PROKR2* mutations accounted for 13.3% (18/135), and W178S mutations accounted for 55.6% (10/18) (41). Combined with these two studies, W178S accounted for 57.1% of *PROKR2* mutations in the Chinese CHH population (20/35). Functional analysis showed that the mutant impeded receptor expression on the cell surface. W178S of *PROKR2* may be an ancient founder mutation, and it was not eliminated in the Chinese CHH population during evolution. The silencing of its effect on reproduction may be related to oligogenicity, or the mutant may revert during adulthood. However, in this study, the mother of 1 patient with the W178S mutation in *PROKR2* was a proband, suggesting that the mutation had a wide spectrum and

that the patient could undergo germ cell maturation after treatment. Therefore, the complex mechanism of its effect on reproduction requires further study.

Of the 5 patients with single-gene mutations in *PROKR2* (W178S), 4 were diagnosed with dual CHH. Some patients harbor additional gene mutations. There was 1 patient with a repetitive mutation site *HS6ST1* (H63D), and another with *IL17RD* (N503S); they were also diagnosed with dual CHH, and *PROKR2* (W178S) and *IL17RD* (N503S) were carried by the proband's mother simultaneously. However, the results of the hCG test showed that the Leydig cells were dysfunctional. It has been suggested that some patients with dual CHH diagnosed by the hCG test in this study may still recover their reproductive function after standard treatment. It could be that Leydig cells had not been stimulated by GnRH for a long time, which may have led to a slow response of the receptor. The curative effects in patients with these mutations and the relationship between the curative effect and gene mutations will be further investigated.

Hypospadias is caused by an abnormal urethral opening closure in the early stage of embryonic development, including the early hormone-independent stage (5–8 weeks) and hormone-dependent stage (8–12 weeks) (42). Before 12 weeks of gestation, the HPG axis has not been activated and cannot secrete gonadotropins, therefore, hypospadias and CHH may be two unrelated diseases. The Europe CHH consensus also believes that the existence of hypospadias could exclude the diagnosis of CHH.

In addition to our previous report, other HH patients with the hypospadias phenotype have also been reported (43–45). We analyzed 7 patients with hypospadias. In **Table 1**, 4 patients (patients 1-3 and 5) carried CHH-related pathogenic genes *HS6ST1*, *ANOS1* and *FGF17* respectively, and these three genes belong to "FGF8 synexpression group". *HS6ST1* encodes a heparan sulfotransferase enzyme, which was required for anosmin-1 function *in vivo*, *ANOS1* could enhance *FGF* signaling by direct physical interactions with the FGFR-FGF-heparan sulfate proteoglycan complex on the cell surface. And *FGF17* has high homology to *FGF8*, while *FGF8* signaling is required for genital tubercle (GT) proximal-distal outgrowth, as abolishing *FGF8* or its receptors leads to GT agenesis in mice, resulting in hypospadias. Patient 4 harbored *CHD7* mutation, and *CHD7* plays an important role in gonad development and signaling, and mutation could cause hypospadias in previous study. Therefore, we believe that although the above genes are the pathogenic genes of CHH, they may also play an important role in the process of earlier penis development, and they may cause hypospadias after mutation, but the specific mechanism needs to be confirmed by further experiments. Two patients (patients 1 and 6) carried *SALL1* and *SPECC1L* mutations at the same time, which are involved in syndromes including hypospadias, but the role of these two mutations in the two patients may need to be further studied. On the other hand, endocrine disrupters like pesticide could also increase the risk of hypospadias (46), the phenomenon of CHH complicated with hypospadias needs more observation and research.

However, there are several limitations in the study. First of all, our olfactory judgment is based on the description and observation of olfaction by children and their parents, rather than olfactory test, some may be confusing. The disadvantage of the study is that only 94 patients (94/125, 75.2%) performed genetic testing, which may underestimate the rate of oligogenicity to CHH. On the other hand, due to the characteristics of CHH disease itself, CHH can only be confirmed after 18 yrs of age. Among our 125 patients, 27.2% are followed up to reach 18 years old, which requires longer follow-up to further verify the diagnosis of these patients.

## CONCLUSION

Micropenis, cryptorchidism, and molecular genetics are important factors for diagnosing CHH in pediatric patients. In this study, 15 new CHH genes were confirmed in patients. Oligogenic mutations accounted for 27.7% of all CHH patients, which may have been a mechanism of autosomal recessive hereditary genes or incomplete gene penetrance pathogenicity. KS due to *ANOS1* mutations is mostly caused by single genes, and *CHD7* mutations lead to isolated CHH. Approximately 25.0%–54.5% of common pathogenic gene mutations are uncertain, and their roles in the pathogenesis of CHH require further study. *PROKR2* and *KISS1R* may also share genes involved in DP and CHH. The *FGF* signaling pathway, represented by *FGFR1*, mainly causes CHH. The mothers of multiple probands carried the same mutation, and multiple gene mutation sites repeatedly appeared, suggesting that the effect of these mutation sites on reproduction was relatively slight. In this study, T after short-term hCG stimulation as an indicator of testicular function may be a false negative. We will further monitor the levels of T and INHB in patients with CHH after GnRH pump or hCG/HMG treatment and discuss dual CHH later.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) repository, accession number CNP0002854.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Beijing Children's Hospital, Capital Medical University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

MQ and LF collected the data. YW analyzed the data and wrote the manuscript. CG revised the manuscript. All authors approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.846801/full#supplementary-material

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# Navigating Disrupted Puberty: Development and Evaluation of a Mobile-Health Transition Passport for Klinefelter Syndrome

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Klinefelter syndrome (KS) is the most common aneuploidy in men and has long-term sequelae on health and wellbeing. KS is a chronic, lifelong condition and adolescents/young adults (AYAs) with KS face challenges in transitioning from pediatric to adult-oriented services. Discontinuity of care contributes to poor outcomes for health and wellbeing and transition programs for KS are lacking. We aimed to develop and test a mobile health tool (KS Transition Passport) to educate patients about KS, encourage self-management and support successful transition to adult-oriented care. First, we conducted a retrospective chart review and patient survey to examine KS transition at a university hospital. Second, we conducted a systematic scoping review of the literature on AYAs with KS. Last, we developed a mobile health transition passport and evaluated it with patient support groups. Participants evaluated the tool using the System Usability Scale and Patient Education Materials Assessment Tool (PEMAT). Chart review identified 21 AYAs diagnosed between 3.9-16.8 years-old (median 10.2 years). The survey revealed only 4/10 (40%) were on testosterone therapy and fewer (3/10, 30%) had regular medical care. The scoping review identified 21 relevant articles highlighting key aspects of care for AYAs with KS. An interprofessional team developed the mobile-health KS transition passport using an iterative process. Support group members (n=35) rated passport usability as 'ok' to 'good' (70 ± 20, median 73.5/100). Of PEMAT dimensions, 5/6 were deemed 'high quality' (86-90/100) and participants knew what to do with the information (actionability = 83/100). In conclusion, many patients with KS appear to have gaps in transition to adult-oriented care. Iterative development of a KS transition passport produced a mobile health tool that was usable, understandable and had high ratings for actionability.

**Keywords: adolescent, continuity of care, puberty, Klinefelter syndrome (KS), transition**

## INTRODUCTION

Klinefelter syndrome (KS, 47, XXY) is the most common chromosomal disorder in males occurring in approximately 150 per 100,000 males (1:660) (1). Klinefelter, Reifenstein, and Albright first described KS in 1942 (2) and subsequent literature has expanded the variable phenotypic features and abnormalities observed in KS. The clinical constellation of small firm testes, primary (hypergonadotropic) hypogonadism, gynecomastia, infertility as well as a range of neuropsychiatric issues and learning disabilities are hallmark signs (3). The most common KS karyotype is 47 XXY and milder mosaic forms may be noted (4). As the degree of testicular insufficiency can be variable, clinical signs are subtle in many affected men limiting early and appropriate diagnosis. Indeed, a marked discrepancy was reported in a large Danish registry study when comparing KS prevalence in male fetus undergoing prenatal testing versus the postnatal diagnosis rate in the general population, suggesting that fewer than 25% of men with KS are diagnosed in a real-life setting (5).

KS can have wide-ranging effects on health and wellbeing (6, 7). The pituitary gonadal axis is disrupted (hypergonadotropic hypogonadism) and can manifest as disrupted puberty. There are striking metabolic disturbances with altered body composition and increased risk for insulin resistance and type 2 diabetes. Bone mineralization may be compromised and a spectrum of neurocognitive functional impairments as well as increased psychological and psychiatric morbidity are observed (6). Effective management requires a multidisciplinary approach that may include endocrinology, andrology/urology, neuropsychology, psychiatry, genetic counseling, nursing, social work, speech and language therapy, occupational therapy, and behavioral specialists (3). Only approximately 10% of cases are diagnosed during childhood (5). Patients diagnosed in childhood may exhibit more pronounced health and cognitive factors that challenge key developmental tasks. For adolescents, KS may affect developing autonomy, self-care skills, and care coordination during the transition from pediatric services to adult-oriented care. For young adults, gaps in care (e.g., "lost to follow-up") can have significant negative sequelae on health and wellbeing (8). Structured approaches to transitional care for young adults with complex medical needs are needed to help patients develop self-management skills and support continuity of care (9). However, there are few published examples of structured transitional care for KS (10, 11). As such, there is limited information on best practices and evidence-based guidelines have yet to be established for KS transitional care.

To address the knowledge gap related to KS transition, we used a three-tiered approach. First, we conducted a retrospective chart review to examine the discontinuity in transitional care for patients with KS. Second, we conducted a systematic scoping review to evaluate the available evidence and recommendations for adolescents and young adults (AYAs) with KS during the transition to adult-oriented care. Third, we developed a novel mobile health (m-health) 'transition passport' to support and guide patients in self-management during transition and to ensure information transfer. Finally, we sought to validate the transition passport with KS patient-support groups by examining usability, understandability, and actionability of the transition passport.

## METHODS

This study was conducted in accordance with the Declaration of Helsinki and was approved by local ethics committees (Lausanne University Hospital Ethics Committee and Boston College Institutional Review Board). All participants provided informed consent prior to study activities. The study was comprised of three parts. First, we conducted a chart review of the Lausanne University Children's Hospital (Switzerland) and surveyed patients about their experiences during transition to adult-oriented care. The results of part I pointed to gaps in care and challenges during transition that spurred us to develop a mobile-health tool to support transition for AYAs with KS. The second part of the study involved a systematic scoping review examining the literature on transitional care for patients with KS. This was used to gather the best available evidence for creating the mobile-health tool. Last, we created a transition passport for patients with KS and validated it with patient support organizations.

### Chart Review and Patient Survey

To better understand the needs of patients with KS during transition we conducted a retrospective, descriptive follow-up study of AYAs with KS (>16 yrs.) seen in the pediatric endocrine service of the Lausanne University Children's Hospital. Patients were identified by searching the hospital database of patients (2000-2014). Patients after 2014 were not included - as a structured transition program (Center for Endocrinology and Metabolism in Young Adults, CEMjA) was launched in 2014 (11). Individual chart review was performed to collect data on timing of diagnosis, medical management, and continuity of care (i.e. medical follow-up). Identified patients were mailed a previously validated questionnaire (12) that was adapted for KS. Briefly, the adapted 32-item instrument included 14 items on patient experiences with KS medical management (and current health), 5 general transition items, 6 KS-specific transition items, and 7 items on how transition could be improved (**Supplemental Material 1**).

### Systematic Scoping Review

To review the data and evidence base related to transitional care for AYAs with KS, we conducted a scoping review (AD, SL) according to the five-stage Arksey and O'Malley framework (13). Findings are reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (14). In brief, the scoping review involved five steps: 1) identifying the research question ("What impact does a structured transition to adult care have on continuity of care, health, and quality of life for young adults with Klinefelter syndrome?"), 2) identifying the relevant literature (systematic literature search using keywords related to KS and transition), 3) selecting the literature (independent investigators screened articles using Rayyan software (15), 4) charting the data (data was extracted from selected articles), and 5) collating, summarizing, and reporting results (evidence summarized in a

table). **Supplemental Material 2** provides a detailed description of methods used for the scoping review of literature (1987-2017). Prior to manuscript preparation, the scoping review was repeated (2017-2021) to identify recently published studies (AD/LE) – reported in **Supplemental Material 3**.

## Transition Passport Development

An interprofessional team including pediatric/adult endocrinology, urology, and nursing engaged in an iterative process to develop content and design a mobile, electronic health (m-health) tool (i.e., KS transition passport) to support AYAs transition from pediatric to adult-oriented care. Broadly, the goal was to create an interactive tool (PDF) organized into easily navigable sections enabling patients to link/jump to preferred topics by clicking on icons - without having to read through the entire document (i.e., traditional printed materials). The KS transition passport serves as a psychoeducational tool enabling AYAs to learn about KS as well as a means to document and record information essential to their medical management. The interactive PDF format was chosen for reasons of simplicity as the format can be used with standard mobile electronic devices and independent of web connectivity and/or linkage to electronic health records. Language editing, word choice, layout, and design elements were modified and refined in an iterative development process. The final version (V.20) was translated from French to English. We assessed readability using algorithms that calculate the number of difficult words/sentences to provide an estimated age range and grade reading level. There is no accepted "gold standard" algorithm to assess readability so we utilized several validated instruments: Automated Readability Index, Linsear Write Formula, Flesch-Kincaid Grade Level, The Coleman-Liau Index, Flesch Reading Ease, and Gunning Fog Index and reported the calculated consensus reading level as previously reported (16).

## Evaluation of Transition Passport

To evaluate the transition passport, we partnered with patient organizations and used a web-based approach to reach geographically dispersed patients (17). Adult patients and parents/guardians able to read/write English were included for the evaluation of the transition passport. After providing electronic opt-in consent, participants were asked to review the transition passport and were informed that the purpose of the m-health tool was to support AYAs during transition to adult-oriented care. Participants viewed a pdf of the transition passport and completed a QualtricsTM survey to record sociodemographic information and several measures. Subjective health literacy was assessed as described by Chew and colleagues (18). This brief instrument has been shown to detect limited health literacy as assessed by lengthier validated instrument (Rapid Estimate of Adult Literacy in Medicine, AUROC=0.82) (19). Objective health literacy was measured using the Newest Vital Sign (NVS) (20). The NVS is a 6-item instrument that is widely used to identify individuals with limited health literacy and numeracy skills. Briefly, individuals are presented with a nutrition label and are required to identify and interpret basic text and perform simple mathematical computations. Scores identify adequate (score = 4-6), possibly limited (score = 2-3), or high likelihood (50%) chance of limited health literacy/numeracy (score = 0-1). The NVS correlates with the lengthier validated instrument (Test of Functional Health Literacy in Adults, AUROC=0.88) (20) and has good internal consistency (literacy: $\alpha$=0.91, numeracy: $\alpha$=0.78) (21).

To assess usability of the transition passport, participants completed the System Usability Scale (SUS) (22). Users respond to 10 questions (alternately worded positively and negatively) and record strength of agreement using a 5-point Likert-type scale. Scores are transformed into an overall score (0-100). The instrument is reliable ($\alpha$=0.85) and is widely used to measure usability and satisfaction. Participants also completed the "gold standard" Patient Education Materials Assessment Tool (PEMAT). The PEMAT is a validated, 17-item instrument developed by the U.S. Department of Health & Human Services Agency for Health Research & Quality to evaluate print/audiovisual educational materials (23). After reviewing the materials, participants select agree/disagree/not-applicable to items assessing six domains relating to understandability (ability to process key messages - i.e. content, style, use of numbers, organization, design, and use of visual aids) and a domain on actionably (ability to identify steps one can take in response to presented information). Cumulative scores are expressed as a percentage (total score/possible total X 100). Psychometric evaluation demonstrates strong internal consistency, good reliability, and evidence of construct validity (23).

## Statistical Analyses

Data are reported using descriptive statistics. Student's T-tests and Mann-Whitney rank sum tests were used (as appropriate) to compare SUS ratings between patients and parents/guardians. PEMAT scores ≥80% on a given parameter were deemed be 'high-quality' (16). Chi square and Fisher exact tests were used as appropriate to compare patients and parents/guardians for subjective/objective health literacy and PEMAT ratings. A $p$ value <0.05 was considered statistically significant.

# RESULTS

## Chart Review and Patient Survey

The retrospective search of the Lausanne University Hospital (CHUV) pediatric endocrine database identified 21 White non-Hispanic patients with KS (2000-2014). Age at initial consultation ranged from 3.9-16.8 years-old (median 10.2 yrs.). Of those responding to the survey, more than half of patients (9/14, 64%) received testosterone therapy in the pediatric setting. The duration of care in pediatrics ranged from 1-11 yrs. (mean 4.5 ± 3.5, median 4 yrs.). A survey was sent to patients and 10/14 (71%) completed the survey. Half of patients (5/10, 50%) reported being clinically followed in adult endocrinology and 3/10 (30%) reported that they did not seek regular medical care. Only 4/10 (40%) had ongoing testosterone treatment. We inquired about the preferred timing of transition and the majority of patients preferred transition between 16-19 yrs. (16-17 yrs.: n=4/10, 18-19 yrs.: 3/10). In terms of the transition process, results were divided. Half of patients (5/10) preferred to see the adult provider alone while the other half preferred to have parents included in medical visits.

## Systematic Scoping Review

To explore the survey findings suggesting gaps in care and challenges with adherence to treatment, we conducted a systematic scoping study of the available literature on AYAs with KS (1987-2017). From the initial review of 134 articles, 22 were included for full review and data extraction. The publications were from Australia, Belgium, France, Netherlands, Switzerland, and the United States. Data were extracted from articles on AYAs with KS including 11 review articles (8, 24–33), two systematic review/meta-analyses (34, 35), 6 cross-sectional/observational studies (36–41) and two interventional studies (42, 43). Most articles (13/21, 62%) were published between 2012-2017, pointing to a growing attention to the care of AYAs with KS. Indeed, following the evaluation of the KS transition passport (below) we repeated the structured literature search for publications 2017-2021 (**Supplemental Material 3**). Notably, there is no "gold standard" approach to treatment for AYAs with KS and evidence-based consensus guidelines are lacking (8, 31). From our initial scoping review, the most frequently addressed topics included psychological aspects of KS, fertility, and puberty. Considerations for bone, metabolic, autoimmune, and hematologic issues were less frequently discussed. Psychological/psychiatric aspects of KS were the most frequently examined (16/21, 76%) in identified articles (8, 24–32, 36, 37, 39–41, 43). A number of articles highlighted the psychosocial impact, health-related quality of life (HR-QoL), depressive symptoms, and adaptation to living with KS. Aspects of care relating to fertility were the second most frequently addressed in articles (13/21, 62%) (8, 28–30, 32–35, 38, 41–43). Infertility treatment (i.e., testicular sperm extraction, sperm retrieval rates, cryopreservation) was the dedicated topic of 6/21 (29%) of publications (33–35, 38, 42, 43). Of note, all (4/4) interventional studies and systematic reviews/meta-analyses focused on infertility treatment. Considerations relating to puberty (and testosterone replacement) were discussed in 9/21 (43%) (8, 25, 28–32, 39, 41), while metabolic (29, 31–33), bone health (8, 31, 32), and autoimmune disorders (8, 31) were less frequently noted. The available evidence/expert opinion of identified articles is charted in **Table 1**.

## Transition Passport Development

Spurred by the findings of the chart review and drawing on scoping review findings, an interprofessional team (endocrinology, urology, nursing) developed a KS transition passport to support effective transition to adult-oriented care. A series of face-to-face meetings charted the structure of the passport (an interactive PDF, **Supplemental Materials 4**, **5**) and topical material for the passport. Each section is matched with a representative icon for visual appeal and to decrease reading burden. Sections include informational content providing a brief overview of KS, chapters dedicated to specific developmental periods (at birth, infancy, childhood, puberty, young-adulthood, adulthood) and a summary of tests with recommended timing/frequency. Additional sections provide interactive components for patients to record information related to their care: 'my follow up' (dates for visits across specialty), 'my treatments' (type and timing), 'my results' (recent test results) and 'my contacts' (information for providers across disciplines and links to patient organizations). After multiple revisions, the final version (V.20) was translated from French to English and a single page 'user guide' was created to provide orientation to the KS transition passport (**Supplemental Material 4**). Assessing the readability of the final version (V.20) (**Supplemental Presentation 5**) revealed a range of readability scores (Automated Readability Index: 8.7 "8th- 9th grade reading level", Linsear Write Formula: 8.9 "9th grade", Flesch-Kincaid Grade Level: 9.7 "10th grade", The Coleman-Liau Index: 11.0 "11th grade", Flesch Reading Ease score: 51.4 "fairly difficult to read", Gunning Fog: 12.8 "hard to read"). The automated consensus score of the algorithms yielded a rating of "fairly difficult to read" - consistent with a 10th grade (14-15 year-old reading level).

## Transition Passport Evaluation

To evaluate the KS transition passport, we recruited subjects through several KS patient support organizations. In total, 35 participants completed the evaluation (**Table 2**). Overall, participants had relatively high levels of education with 27/35 (77%) having a college education or higher. Both subjective and objective health literacy were similarly high with >90% having adequate literacy/numeracy. No differences were observed between patients and parents/guardians in terms of objective health literacy/numeracy measures (NVS: p=0.37). Overall, participants rated usability (as assessed by the System Usability Scale) as 'okay' to 'good' (mean: 70 ± 20, median: 73.75) and ratings were similar between patients and parents/guardians (p=0.09) (**Figure 1**). We used the 'gold standard' Patient Education Materials Assessment Tool to assess understandability and actionability of the KS transition passport (**Figure 2**). Specific dimensions with scores >80 are considered 'high-quality'. Five of six dimensions relating to understandability received scores >80 (range: 86-90/100). The 'use of visual aids' scored 79/100 and was the only PEMAT dimension below the *a priori* threshold of 80. Understanding what to do with the information (actionability) was rated 83/100. Patients with KS and parents/guardians exhibited similar PEMAT scores ($p$=0.9) – yet this observation should be interpreted with caution given the limited number of parent/guardian participants.

# DISCUSSION

This three-part study provides several important insights into the management of adolescents and young adults (AYAs) with KS. First, our retrospective chart review indicated that a significant portion of young men with KS struggled with transitioning from pediatric to adult-oriented care resulting in gaps in treatment and care. Second, the systematic review of the literature and scoping study highlighted a lack of consensus regarding transitional care of AYAs with KS. The paucity of high-quality data limits the development of evidence-based guidelines for practice. We developed a KS transition passport using an iterative process with an interprofessional team incorporating findings form the scoping review. Evaluating the KS transition passport with patient groups showed that the passport is usable, understandable, and actionable – and thus, may represent an important avenue for extending the reach of care for AYAs with KS.

**TABLE 1 |** Summary of findings from the systematic scoping review (n=21, 1987-2017).

| ref # | year | design | pub. | fertil. | psych. | metab. | bone | Imm. | Summary of findings [country] |
|---|---|---|---|---|---|---|---|---|---|
| (24) | 1989 | review | – | – | YES | – | – | – | [USA] n=16 studies (only 1 specifically on AYAs), increased timidity, low confidence, late development of sexual interest, severe psychiatric illness is rare |
| (25) | 2002 | review | YES | – | YES | – | – | | [USA] KS variable, most do not differ significantly from peers, intelligence is normal, problems may include low muscle tone, poor coordination, speech delays, low self-esteem, delayed sexual interest, initiation of TRT is important for AYAs |
| (26) | 2004 | review | – | – | YES | – | – | – | [USA] Only general information (not specific to KS), general 'good clinical practices' for psychological support during transition |
| (27) | 2004 | review | – | – | YES | – | – | – | [USA] Internalizing (anxiety, depression, withdrawal), social/emotional difficulties, inhibition/Bx issues, language-based learning difficulties, dyslexia, ADD/ADHD, learning disability, executive dysfunction. |
| (36) | 2006 | observational | – | – | YES | – | – | – | [Australia] n=32 (median: 24.5 yrs. [13–45]), 53% on TRT, 34% with normal T levels, 69% had psychosocial issues, 28% had impulse control issues, frequently lost to F/U with psychiatric services, TRT associated with improved mood, less depressive symptoms |
| (28) | 2010 | review | YES | YES | YES | – | – | – | [USA] General overview of TC and barriers, KS specific aspects include: TRT, infertility, T2DM, low BMD, breast/mediastinal cancers, and psychological support for learning difficulties and low self-esteem |
| (29) | 2011 | review | YES | YES | YES | YES | – | – | [USA] hypogonadism often appears in AYAs, cryptorchidism (5-69%), increased fat mass common, role of TRT on neuropsychological status is unclear |
| (37) | 2011 | observational | – | – | YES | – | – | – | [USA] n = 310 (40.7±14 yrs. [14-75]), 69% had significant depressive symptoms (CES-D), depressive symptoms were significantly associated with emotion-focused coping strategies/ perceptions of stigmatization, perceived negative consequences of KS (IPQ), and infertility |
| (34) | 2012 | systematic review | – | YES | – | – | – | – | [USA] n=16 studies, overall 51% SRR (better rates with micro-TESE), no recommended age for SRR, positive SRR predictors = age <35yrs., near-normal T levels, response to hCG pre-op; negative predictors = low T at Dx |
| (38) | 2012 | observational | – | YES | – | – | – | – | [Belgium] n = 7 (13-16 yrs.), all biopsies showed IF, SPGA found in 1/7, authors propose testicular tissue preservation ideally be done before adolescence (yet no evidence on fertility outcomes). |
| (30) | 2014 | review | YES | YES | YES | – | – | – | [France] cryptorchidism increased in KS, testicular growth ends in mid-puberty, 15yrs. appears reasonable as lower limit for fertility preservation given psychosexual maturity, prior reports of increased psychiatric disease/mental retardation/criminality have not been confirmed by longitudinal studies |
| (8) | 2014 | review | YES | YES | YES | YES | YES | YES | [Belgium] multidisciplinary approach is important, high risk for lost to F/U, screen T in adolescence and initiate TRT as needed, SA can begin at 14yrs., screen AYAs for MetS/ T2DM and associated diseases (autoimmune), begin DXA screening at end of puberty, increased VTE risk with KS, psychosocial support and focus on self-esteem during TC |
| (31) | 2015 | review | YES | YES | YES | YES | YES | YES | [Switzerland] Review of multiple hypogonadal states, importance of structured TC, puberty is normal in most KS, hypogonadism first evident in AYAs, increased risk for MetS, T2DM, decreased BMD, some autoimmune conditions, disrupted puberty can have psychological burden (victimization/bullying), provide anticipatory guidance/emotional support and openly discuss patient concerns during TC |
| (39) | 2015 | observational | YES | – | YES | – | – | – | [USA] n = 43 (8-18 yrs.), 70% had learning difficulties, 67% had speech & language problems, 63% had social interaction problems, 67% had impaired HR-QoL, 38% had low self-esteem, 26% had poor selfconcept, increased risk for depression, T levels not associated with psychosocial health measures |
| (40) | 2015 | observational | – | – | YES | – | – | – | [USA] n=310 (40.7 yrs. [14-75]), 76% had significant negative consequences of KS (IPQ), 69% had significant depressive symptoms (CES-D), 64% had high levels of adaption, 56% used emotion focused coping strategies, use of problem-focused coping strategies was the greatest predictor of adaptation, reframing cognitive appraisals may promote problem-focused coping and improve adaptation |
| (42) | 2015 | interventional | – | YES | – | – | – | – | [France] n=41 (15-22yrs: n=16, >16yrs: n=25), recommend TESE be discussed/offered (<35yrs.), overall SRR 50%, unclear evidence regarding timing of D/C TRT before TESE, 10 studies report SRR in AYAs (success: 0-70%), SRR higher for boys >15yrs., 8% with non-mosaic KS had sperm in the ejaculate |
| (43) | 2016 | interventional | – | YES | YES | – | – | – | [USA] n=28 (12-25yrs: n=15), 10/15 (66%) AYAs underwent micro-TESE, SRR rate 50%, no association between SRR and hormonal markers or TV, significantly more overall difficulties/symptoms (SDQ), 60% had an IEP, 40% had received MH services, 27% had ADD |
| (32) | 2016 | review | YES | YES | YES | YES | YES | – | [USA] most AYAs with KS enter puberty normally, TRT should be prescribed per ES guidelines, |

*(Continued)*

**TABLE 1** | Continued

| ref # | year | design | pub. | fertil. | psych. | metab. | bone | Imm. | Summary of findings [country] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | infertility is common in KS, SRR by TESE 54%, SRR optimal during peak Leydig cell function, decreased BMD |
| (33) | 2016 | review | – | YES | – | – | – | – | [Belgium/USA] pro/con debate, limited data available, SPGA stem cell/testicular tissue freezing/*in vitro* maturation strategies require further validation, testicular tissue from AYAs is controversial and should only be performed in a research framework, many ethical considerations |
| (35) | 2016 | systematic review | – | YES | – | – | – | – | [Netherlands] n=76 studies, KS often Dx in childhood due to Bx issues vs. infertility in adults, overall SRR rates are low for AYAs (n=10 studies, 0-70%), pre-pubertal TESE should not be offered (psychological impact), counselling should be provided in advance of SRR/TESE, 4 studies showed no spermatozoa in AYA ejaculate (10-25yrs: n=56). SRR higher for AYAs >15 yrs., only SPGA found were in children, 8% of non-mosaic KS have sperm in ejaculate, overall SRR in KS is 50% |
| (41) | 2017 | observational | YES | YES | YES | – | – | – | [USA] N = 310 (14-24yrs : n=31), Age and time of Dx were not predictive of psychological well-being, social impact similar in AYAs and adults, 21% said "worst" part of KS were small TV/gynecomastia/ height/low muscle mass due to victimization/bullying, 31% said infertility was the greatest challenge, 31% said psychological impact of KS Dx was the greatest challenge, 23% reported learning difficulties, 22% reported social problems, disclosing Dx to others was challenging but ultimately strengthened relationships and supported adaption to life with KS |

*headers Pub, puberty; Fertil, fertility; Psych, psychological/psychosocial; metab, metabolism; Imm, autoimmune disorders; table ADD/ADHD, attention deficit disorder/attention deficit hyperactivity disorder; AYAs, adolescents and young adults; BMD, bone mineral density; Bx, behavior; CES-D, Center for Epidemiologic Studies Depression; Dx, diagnosis; DXA, dual X-ray absorptiometry; ES, Endocrine Society; F/U, follow-up; hCG, human chorionic gonadotropin; HR-QoL, health-related quality of life; IEP, individualized educational program; IF, interstitial fibrosis; IPQ, Illness Perceptions Questionnaire; MetS, metabolic syndrome; MH, mental health; micro-TESE, microdissection testicular sperm extraction; SDQ, Strengths and Difficulties Questionnaire; SPGA, spermatogonia; SRR, sperm retrieval rate; T, testosterone; T2DM, type 2 diabetes mellitus; TC, transitional care; TESE, testicular sperm extraction; TRT, testosterone replacement therapy; TV, testicular volume; VTE, venous thromboembolism.*

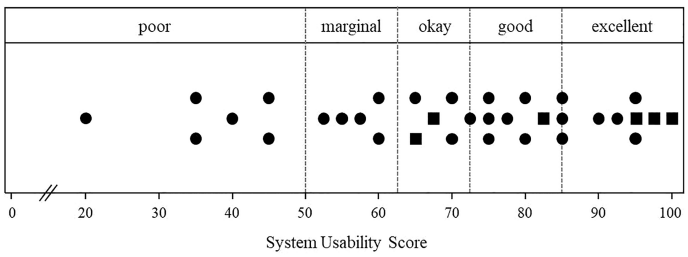
Given the broad phenotypic spectrum of KS, some AYAs with KS may not differ significantly from their peers. Overt KS phenotype is often evident only after puberty or when fertility is pursued (25, 44). Subtle clinical presentation or mosaicism (4) may contribute to the estimated 75% of men with KS who remain undiagnosed (5). It is plausible to consider that earlier KS diagnosis is likely in more severely affected individuals. The present data support the notion that there are often gaps in transition. Such discontinuity of care can affect health outcomes and quality of life and may contribute to avoidable healthcare costs. A 2016 Cochrane review on transition of care for AYAs with special health needs concluded that effective transitional care has several key benefits including enhanced self-management as well as improved patient outcomes, satisfaction, and adherence to treatment (45). For AYAs with KS, gaps in care can have detrimental effects on sexual, bone, and metabolic health and can contribute to impaired health-related quality of life (8, 31). There is growing attention to the importance of transitional care for AYAs with KS as demonstrated by our initial scoping review in which 13/21 (62%) were published between 2012-2017. Further, the updated scoping review following evaluation of the KS transition passport identified 23 articles (2017-2021) supporting growing focus on AYAs and transitional care in recent years.

**TABLE 2** | Demographic characteristics of participants evaluating the KS transition passport (n=35).

| Characteristic | n (%) |
|---|---|
| **Participants** | |
| patients | 29 (83%) |
| age (yrs): mean±SD (median, range) | 47.9±17.7 (50, 21-77) |
| age at diagnosis*: mean±SD (median, range) | 28.0±14.5 (29, 1-61) |
| parents/guardians | 6 (17%) |
| age (yrs): mean±SD (median, range) | 58.3±16.8 (64, 20-77) |
| **education** | |
| less than college (high school/vocational) | 8 (23%) |
| college | 16 (46%) |
| post-graduate | 11 (31%) |
| **subjective health literacy†** | |
| adequate | 32 (91%) |
| inadequate | 3 (9%) |
| **objective health literacy – NVS** (n=25) | |
| adequate | 23 (92%) |
| moderate | 2 (8%) |
| low | 0 |

**three patients diagnosed prenatally and were not included in the calculation.*
*†subjective health literacy (18). NVS: Newest Vital Sign (20).*

To support AYAs during transition (i.e., 16-25 yrs.), an interprofessional team developed the "KS transition passport". The m-health tool was intended to serve as a psychoeducational support for therapeutic education to help patients better understand KS, as well as a resource promoting patient activation (i.e., empowerment) enabling patients to record vital health information during the transfer from pediatric to adult-oriented care. The passport can equally be used as a standardized information transfer tool between health professionals. The idea of using a "passport" to support AYAs during transition is not new.




| group | range | median | mean ± SD |
|---|---|---|---|
| patients (n=29) | 20 - 100 | 72.5 | 68.2 ± 20.4 |
| parents/guardians (n=6) | 67.5 – 97.5 | 86.25 | 83.8 ± 14.3 |
| total (n=34) | 20 - 100 | 73.75 | 70 ± 20 |

**FIGURE 1** | System Usability Scale (SUS) ratings of the KS transition Passport (n=34). Individual SUS ratings are shown for patients (circles) and parents/guardians (squares). Cutoffs are depicted by dotted lines. The table reports descriptive statistics of patient, parent/guardian and total ratings. Patient and parent/guardian SUS scores did not differ (p=0.094).

Indeed, the Lausanne University Children's Hospital has developed and uses a transition passport for type 1 diabetes and Turner Syndrome. Other groups have published findings on similar passports for AYAs with diabetes (46), inflammatory bowel disease (47), congenital heart defects (48, 49), and osteogenesis imperfecta (50). While the KS Transition Passport is not an intervention to specifically improve physical and intellectual development, we envisioned it as a psycho-educational tool to enhance patient awareness and understanding of their condition and to support patient activation (i.e., PEMAT actionability domain). Notably, such patient activation is critical for empowering individuals for self-management of a chronic health condition and helps contribute to continuity of care as well as and improved health outcomes (51). We evaluated the KS transition passport with KS patient support organizations and found the usability of the eHealth tool was 'good'. However, 6/34 (17.6%) respondents rated usability as 'poor'. Half of those giving a 'poor' rating reported their highest level of education as vocational training – yet 6/6 had adequate subjective health literacy and 5/6 had adequate objective health literacy scores. Thus, a 'poor' usability rating does not appear to be linked to health literacy and additional usability enhancements could be made. In terms of the PEMAT, 5/6 dimensions assessing understandability and actionability (i.e., knowing what to do with the information) were considered 'high-quality' (i.e., >80%) suggesting high acceptability ratings. Similar to other studies evaluating transition passports, our findings provide initial support for the KS transition passport as a promising complement to structured transitional care for AYAs with KS.

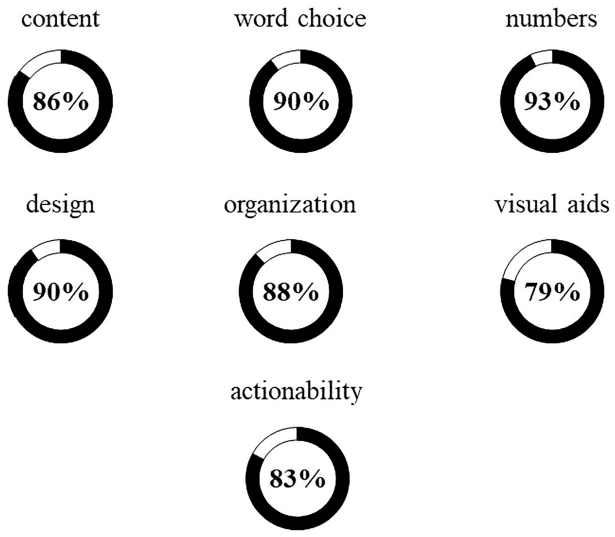



**FIGURE 2** | Patient Education Materials Assessment Tool (PEMAT) ratings of KS transition passport understandability and actionability (n=35). The PEMAT includes 17 items within seven domains. Six domains relate to understandability (top two rows) and the other is actionability. Approval ratings are depicted by circle areas in black and percent approval is noted in the center for each domain. Ratings ≥80% are considered 'high-quality'. Patient and parent/guardian PEMAT scores did not differ.

Relative strengths of the study include the sequential process (i.e., retrospective chart review, systematic scoping review, iterative tool development and evaluation using 'gold standard' measures). The study has a number of limitations including the relatively small sample size for the retrospective chart review/survey (n=21) and the evaluation with KS patient organizations (n=35). We did not collect data on self-identified race/ethnicity. As such, the findings should not be considered to represent the perspectives of individuals with Klinefelter syndrome from diverse ancestry. Indeed, a recent review has identified noted paucity of data on the experiences patients with Klinefelter syndrome from and Black, Indigenous, People of Color (BIPOC) communities (52). Similarly, we did not capture whether or not individuals were treated with testosterone in the pediatric setting and cannot make any inference on the potential role of treatment on the evaluation of passport. It is worthwhile to note that the participants had high levels or health literacy/numeracy and may not be representative of all patients and parent/guardians. Patient-facing materials are recommended to be written at the 6-8th grade reading level (i.e., 11-13 yrs.).

Despite the iterative process guided by principles of health literacy 51, we were only able to achieve a 10th grade (14-15 year-old) reading level. This is likely due to the fact that several complex, multisyllabic words needed to be stated and defined (e.g., chromosome, Klinefelter, testosterone, azoospermia, metabolic, osteoporosis). However, as we considered transition to encompass 16-25 years-old, the 14-15 year-old reading level of the passport may not overly be problematic. We did not observe any differences in PEMAT scores between patients and parents/guardians - yet this finding should be interpreted with caution given the limited number of participants. The mean age of diagnosis of KS patients evaluating the transition passport was 28 years-old and these men may have milder KS phenotypes.

The iterative development process involved an interprofessional team yet did not involve patient/parent stakeholders. This is relevant as involving patient stakeholders in a "design-thinking" (i.e., user-centered design) process can improve understandability, actionability and acceptability of co-created patient-facing materials 16. Almost a third (11/35, 31%) of patient/parents provided free-text comments that were generally positive and expressed appreciation for the work. However, a number of comments also noted a desire for greater detail regarding mosaicism, thrombosis risk, complexity of behavioral issues, how to find a specialist/ask for referrals, and starting an occupation. Indeed, comments from patients/parents requesting information and greater detail on learning difficulties, academic supports, disability/function, and psychiatric diagnoses may reflect that behavioral health specialists (e.g., psychologists/psychiatrists, professionals in special education) were not involved in the iterative 'design thinking' process. In hindsight, inclusion of such perspectives could have strengthened the development of the m-health tool. Notably, patient-facing material need to be regularly reviewed and updated to ensure that the information accurately reflects the current state of the science and standards of care as well as unmet patient needs. Future work should include systematic follow up of AYAs following transition to adult-oriented care. Additionally, establishing rigorous criteria to identify uniform groups of patients with Klinefelter syndrome (i.e., clinical and molecular features) could support more targeted and rigorous clinical and fundamental research on Klinefelter syndrome.

In conclusion, many patients with KS appear to have gaps in transition to adult-oriented care. Iterative development of a KS transition passport produced a tool that was usable, understandable, and had high ratings for actionability. Such eHealth tools can support a comprehensive, structured approach to transitional care for AYAs with KS. Future work may examine the effect of the KS transition passport on knowledge, patient activation, adherence to treatment, and continuity of care.

## 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 Lausanne University Hospital Ethics Committee and Boston College Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

AD made substantial contributions to the study design, acquired and analysed the literature for the scoping review, contributed to the development of the transition passport, collected and analysed the data for the evaluation of the transition passport and drafted the manuscript. VH made substantial contributions to collecting and analysing the chart review and patient survey data, contributed to the development of the transition passport, provided critical feedback on the manuscript and approved the final manuscript. SL made substantial contributions acquiring and analysing the literature for the scoping review, provided critical feedback on the manuscript and approved the final manuscript. LE made substantial contributions to collecting and analysing the data for the evaluation of the transition passport, provided critical feedback on the manuscript and approved the final manuscript. GP, LV, and NP made substantial contributions to developing the transition passport, provided critical feedback on the manuscript and approved the final manuscript. MH made substantial contributions to the study design, led the development of the transition passport, provided critical feedback on the manuscript and approved the final manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.909830/full#supplementary-material

**Supplemental Material 1** | Patient survey.

**Supplemental Material 2** | Scoping Review methods and PRISMA diagram (1987-2017).

**Supplemental Material 3** | Scoping Review (2017-2021) references and PRISMA diagram.

**Supplemental Material 4** | Passport instructions.

**Supplemental Material 5** | Klinefelter syndrome transition passport.

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# Integrated analysis of proteomics and metabolomics in girls with central precocious puberty

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**Background:** Central precocious puberty (CPP) is a multifactorial and complex condition. Traditional studies focusing on a single indicator cannot always elucidate this panoramic condition but these may be revealed by using omics techniques.

**Objective:** Proteomics and metabolomics analysis of girls with CPP were compared to normal controls and the potential biomarkers and pathways involved were explored.

**Methods:** Serum proteins and metabolites from normal girls and those with CPP were compared by LC-MS/MS. Multivariate and univariate statistical analysis were used to identify the differentially expressed proteins (DEPs) and differentially expressed metabolites (DEMs). Functional annotation and pathway enrichment analysis were performed by using GO and KEGG databases, and candidate markers were screened. Finally, bioinformatic analysis was used to integrate the results of proteomics and metabolomics to find the key differential proteins, metabolites and potential biomarkers of CPP.

**Results:** 134 DEPs were identified in girls with CPP with 71 up- and 63 down-regulated, respectively. Up-regulated proteins were enriched mainly in the extracellular matrix, cell adhesion and cellular protein metabolic processes, platelet degranulation and skeletal system development. The down-regulated proteins were mainly enriched in the immune response. Candidate proteins including MMP9, TIMP1, SPP1, CDC42, POSTN, COL1A1, COL6A1, COL2A1 and BMP1, were found that may be related to pubertal development. 103 DEMs were identified, including 42 up-regulated and 61 down-regulated metabolites which were mainly enriched in lipid and taurine metabolic pathways. KGML network analysis showed that phosphocholine (16:1(9Z)/16:1(9Z)) was involved in arachidonic acid, glycerophospholipid, linoleic acid and α-linolenic acid metabolism and it may be used as a biomarker of CPP.

**Conclusions:** Our study is the first to integrate proteomics and metabolomics to analyze the serum of girls with CPP and we found some key differential proteins and metabolites as well as a potential biomarker for this condition.

Lipid metabolism pathways are involved and these may provide a key direction to further explore the molecular mechanisms and pathogenesis of CPP.

KEYWORDS

central precocious puberty (CPP), proteomics, metabolomics, lipid pathway, biomarker

# Introduction

Sexual development in humans is a continuous process governed by certain rules. Central precocious puberty (CPP) in girls is caused by the early initiation of the hypothalamic-pituitary-gonadal axis (HPGA), which is characterized by rapid development of internal and external reproductive organs and secondary sexual characteristics before the age of 8, and the sequence of sexual development is basically the same as that of normal girls (1). Based on epidemiologic data, there has been a worldwide trend towards earlier onset of puberty and there is an increased incidence of precocious puberty (2–4). Interestingly, the incidence of precocious puberty has also increased during the lockdown due to the COVID-19 pandemic (5, 6). Apart from secondary CPP, the etiology and pathogenesis of this idiopathic condition are still unclear.

It is now widely accepted that the timing of puberty in humans is determined by complex interactions, including genetics, epigenetics, environmental, nutritional and the gut microbiome (7). The monogenic etiologies of CPP puberty that have been described so far include activating mutations in the KISS1/KISS1R system and inactivating mutations in the imprinted genes, MKRN3 and DLK1 (8). Although mutations in KISS1 and KISS1R had been previously identified (9, 10), no other CPP cases have been reported since 2010, suggesting these remain rare monogenic causes of this condition (11). CPP has a genetic predisposition, and mutations in MKRN3 are common monogenic causes of familial CPP (12). Inactivating of mutations in DLK1 are also associated with familial CPP (13), but these are not common monogenic causes (11). In addition to its monogenic etiology, obesity (14) and environmental endocrine disrupting chemicals (EDCs) (15) are currently considered to be the main causes of CPP. EDCs could regulate the activation of either gonadotropin-releasing hormone (GnRH) neurons or gonadal steroidogenesis in order to initiate puberty through epigenetic mechanisms (16). Recently, Heras et al. (17) revealed that linking kisspeptin, hypothalamic paraventricular nucleus (PVN) ceramide synthesis and sympathetic innervation in the rat ovary were key to obesity-induced pubertal precocity. Although more and more studies have attempted to explain the occurrence of CPP, the regulatory network involved in puberty remains a mystery.

In recent years, with the rapid development of post-genomic techniques, the integration of multi-omics data has been widely used to understand complex diseases (18). Proteomics is a science that studies the protein composition and changes that occur in cells, tissues and whole organisms, and this was first proposed by Wilkins in 1996 (19). Metabolomics is an emerging field that provides a comprehensive coverage of biological processes and metabolic pathways by providing a large number of metabolite analyses (20). Therefore, an integrated approach that combines proteomics and metabolomics can be a potentially very powerful tool that can provide an advisable strategy for exploring biomarkers and the molecular mechanisms of diseases. CPP is a disease with complex etiology and pathogenesis, and a reliance on traditional research methods cannot meet the all needs of research into this condition. In our study, we performed an integrated proteomics and metabolomics analysis in order to explore the pathways that were altered in girls with CPP and to reveal potential biomarkers that can aid in its diagnosis.

**Abbreviations:** CPP, central precocious puberty; DEPs, differentially expressed proteins; DEMs, differentially expressed metabolites; HPGA, hypothalamic-pituitary-gonadal axis, EDCs, endocrine disrupting chemicals; COVID-19, coronavirus disease-19; KISS, kisspeptin; PVN, hypothalamic paraventricular nucleus; PCA, principal component analysis; OPLS-DA, orthogonal partial least-squares-discriminant analysis; VIP, variable importance in the projection; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; DIA, data-independent acquisition; CNS, central nervous system; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, false discovery rate; BP, biological process; CC, cellular component; MF, molecular function; PPI, protein-protein interaction; S1P, sphingosine-1-phosphate.

# Materials and methods

## Ethics

This study conformed to the Declaration of Helsinki and was approved by the Scientific Ethics Committee of The First Affiliated Hospital of Guangxi Medical University in Nanning, China (2022 (KY-E-025)). Informed consent was obtained from all participants or their legal guardians.

## Patients

This study was conducted in the First Affiliated Hospital of Guangxi Medical University from May to December 2021. 10 girls with CPP and 10 age-matched prepubertal healthy girls were selected for data independent acquisition (DIA) quantitative proteomics. In addition, 31 girls with CPP and 19 age-matched prepubertal healthy girls were selected for untargeted metabolomics. Among them, proteomic and metabolomic tests were performed on 10 girls with CPP and 10 age-matched controls, and all the data were analyzed jointly. Anthropometric, sexual development and gonadal hormone assessments were collected for all subjects (Table 1). With respect to the criteria for inclusion of the CPP cases, this was referred to the Consensus on diagnosis and treatment of CPP, (2015) (1) and these included: 1) Breast development before 8 years of age; 2) Linear growth acceleration where the annual growth rate was higher than in normal girls; 3) Progressive bone age was more than one year of the chronological age; 4) The pelvic ultrasound of the uterus was 3.4 ~ 4.0cm in length with an ovarian volume of 1 ~ 3mL and the presence of multiple follicles ≥4mm in diameter; 5) The peak level of luteinizing hormone (LH peak) ≥ 5 IU/L and the LH peak/follicle-stimulating hormone (FSH) peak ≥0.6 after GnRH simulation. Girls with CPP but with central nervous system (CNS) abnormalities and other secondary diseases, such as congenital adrenal hyperplasia, McCune-Albright syndrome, and primary hypothyroidism, were excluded from the study.

## Serum samples collection

Venous blood samples were collected early in the morning after fasting for at least 8 hours and placed in a dry blood collection tube. The samples were then centrifuged at 4244g at 4°C for 10 minutes. Serum samples were collected and stored at -80°C. Serum samples of girls with CPP were collected before initiation of GnRHa treatment.

## Proteomics

In our study, serum samples from 20 subjects (10 girls with CPP and 10 controls) were analyzed by DIA quantitative proteomics analysis. The total protein in the sample was extracted and some were used to determine the protein concentration as well as for SDS-PAGE, and the rest was subjected to trypsin digestion. After desalting, LC-MS/MS was used to identify the peptides in the samples. Firstly, a protein spectrum library was established by using the traditional data dependent acquisition (DDA) method, and then the mass spectrometry data of each sample were obtained by using DIA technology. Spectronaut pulsar software was used to search all the raw data thoroughly against the known protein databases (UniProtKB). A database search was performed specifically for trypsin digested samples. Alkylation of cysteine was considered as a fixed modification during the database search. The proteins, peptides and peptide-to-spectrum matched false discovery rate (FDR) were all set to 0.01. For DIA data, the quantification of FDR was set to 0.05 and

TABLE 1 Anthropometric and hormone characteristics of the research subjects in this study.

| Items | | Proteomics | | | | | | Metabolomics | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CPP (n=10) | | | Control (n=10) | | P | CPP (n=31) | | | Control (n=19) | | P |
| Age (y) | | 8.57 ± 0.77 | | | 8.46 ± 0.79 | | 0.756 | 8.17 ± 0.91 | | | 7.88 ± 0.93 | | 0.274 |
| Tanner's stage | Breast | II | III | IV | I | II | | II | III | IV | I | II | |
| | | 4 (40%) | 5 (50%) | 1 (10%) | 9 (90%) | 1 (10%) | – | 20 (64.5%) | 8 (25.8%) | 3 (9.7%) | 17 (89.5%) | 2 (10.5%) | – |
| | Pubic hair | I | II | | I | | – | I | II | III | I | | – |
| | | 7 (70%) | 3 (30%) | | 10 (100%) | | – | 26 (83.9%) | 4 (12.9%) | 1 (3.2%) | 19 (100%) | | – |
| Height (cm) | | 132.15 ± 7.45 | | | 126.80 ± 5.87 | | 0.091 | 132.87 ± 6.93 | | | 124.66 ± 5.36 | | <0.001 |
| Weight (kg) | | 30.65 ± 5.89 | | | 24.69 ± 2.19 | | 0.012 | 28.36 ± 5.18 | | | 23.69 ± 3.34 | | 0.001 |
| Height z score | | 0.04 | | | 0.03 | | – | 0.05 | | | 0.03 | | – |
| BMI (kg/m2) | | 17.38 ± 1.72 | | | 15.37 ± 1.09 | | 0.007 | 16.15 ± 1.56 | | | 15.22 ± 1.65 | | 0.054 |
| BMI z score | | 0.10 | | | 0.08 | | – | 0.09 | | | 0.12 | | – |
| B-LH (IU/L) | | 2.85 (1.25, 6.20) | | | 0.06(0.03, 0.12) | | <0.001 | 1.87 (0.70, 4.09) | | | 0.06 (0.03, 0.08) | | <0.001 |
| P-LH(IU/L) | | 46.26 (14.73, 78.32) | | | – | | – | 20.27 (11.52, 59.97) | | | – | | – |
| B-FSH (IU/L) | | 4.46 (3.55, 5.27) | | | 1.23 (1.05, 1.84) | | <0.001 | 4.65 (3.15, 5.96) | | | 1.32 (1.10, 1.77) | | <0.001 |
| P-FSH(IU/L) | | 17.38 (13.66, 27.46) | | | – | | – | 17.21 (14.16, 24.12) | | | – | | – |
| E2 (pmol/L) | | 40.17 (25.01, 61.37) | | | 26.87 (19.39, 32.87) | | 0.105 | 37.18 (29.23, 55.49) | | | 25.01 (19.49, 28.63) | | 0.002 |

Values are expressed as the means ± standard deviations for normally distributed variables or expressed as medians (interquartile ranges) for non-normally distributed variables. B- LH, basal luteinizing hormone; B-FSH, basal follicle stimulating hormone; E2, estradiol; P-LH, peak luteinizing hormone; P-FSH, peak follicle stimulating hormone.

the quantity MS-level was set at MS2. The differentially expressed protein (DEPs) were identified using the following criteria: 1) Fold change>1.2 or <0.83; 2) p value<0.05 (t-test between the two groups). DEPs were submitted to the DAVID website (https://david.ncifcrf.gov/home.jsp) for functional annotation. Terms of biological processes, cellular component and molecular function were analyzed according to the Gene Ontology (GO) database (http://amigo.geneontology.org) and functional pathway analysis was based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) (21–23) database (http://www.kegg.jp/). Hypergeometric distribution test was used to determine the significance of DEP-enrichment in each GO term or KEGG pathway. A pathway enrichment test p value of less than 0.05, and proteins counts of more than 5 were set as the screening criteria for results.

## Metabolomics

Serum samples from 50 subjects (31 girls with CPP and 19 controls) were analyzed by untargeted metabolomics. A Dionex U3000 UHPLC ultra high performance liquid series QE PLUS high resolution mass spectrometer, composed of liquid-mass coupling system was used for analysis of samples. Progenesis QI software (Waters Corporation, Milford, USA) was used to analyze the LC-MS raw data. Three-dimensional datasets such as m/z, peak RT and peak intensity were formed into an Excel file, and RT–m/z pairs were used as the identifiers of each ion. The resulting matrix was further reduced by removing any peaks with missing values (ion intensity=0) in more than 50% of the samples. An internal standard was used for data quality control (QC; reproducibility). QC samples were prepared by mixing aliquots of all the samples into a pooled sample. The metabolites were identified by using Progenesis QI Data Processing Software, based on publically available databases such as http://www.hmdb.ca/ ; http://www.lipidmaps.org/ as well as self-built databases. The positive and negative data were combined to obtain a combined dataset which was imported into the R ropls package. Principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) were used to observe the metabolic alterations between the two groups, after mean centering (Ctr) and Pareto variance (ParV) scaling, respectively. Variable importance in the projection (VIP) ranked the overall contribution of each variable to the OPLS-DA model. To prevent overfitting of the model, 7-round cross-validation and 200 response permutation testing (RPT) were used to evaluate the quality of the model. Metabolites with VIP values larger than 1.0 and p values less than 0.05 were considered to be DEMs between the groups.

## Statistical analysis

Statistical analysis was performed by using SPSS Statistics Version 23.0 software (IBM SPSS, Armonk, NY, USA). Data was tested for normality of distribution by using the Shapiro–Wilk test. Clinical variables were compared between groups using t-test for normally distributed data, and the Mann-Whitney U test was used for non-normally distributed data. R software (version 3.6.2) was used for statistical analyses. The ropls package analysis software was used for multivariate statistical analysis (PCA, OPLS-DA and OPLS). Analysis packages included pheatmap, ggplot2 and ggrepel and correlation analysis was performed using corrplot. Univariate statistical correlation was performed by using a basic software package. Statistical significance was defined as a p value equal to or less than 0.05.

# Results

## Proteomic analysis

Our study identified 10009 peptides and quantified 1002 proteins. Of these, 134 quantified proteins were identified as DEPs, of which 71 were up-regulated and 63 were down-regulated in the CPP group when compared to the normal group (>1.2-fold or <0.83-fold). The 134 DEPs were visualized by using a volcano plot as shown in Figure 1.

After the DEPs were obtained, GO/KEGG enrichment analysis was performed to describe their functions. The subcellular localization of 134 DEPs were analyzed. The top three subcellular localization were extracellular (48.5%), cytoplasmic (17.2%) and nuclear (16.4%) as shown in Figure 2A. Then, all the DEPs were classified into biological process (BP), cellular component (CC) and molecular function (MF) based on three probable functions. For the up-regulated proteins, the top three of BP were extracellular matrix organization, cell adhesion and cellular protein metabolic process (Figure 2C, green label). The top three of CC were extracellular exosome, extracellular region and extracellular space (Figure 2C, blue label). This is consistent with the subcellular localization analysis which showed that 48.5% of the DEPs were located extracellularly (Figure 2A). The top ten of MF were mainly related to binding functions (Figure 2C, red label). For the down-regulated proteins, the top three of BP were immune response, innate immune response and complement activation *via* the classical pathway (Figure 2D, green label). The top three of CC (Figure 2D, blue label) and the top ten of MF (Figure 2D, red label) were similar to those of the up-regulated proteins. The top 20 of the KEGG enrichment pathways based on the DEPs between girls with CPP and the normal group are shown in Figure 2B (see the supplement datasets for all the significant terms). ECM-receptor interaction, Fc gamma R-mediated phagocytosis, focal adhesion, PI3K-Akt signaling pathway, phagosome and proteoglycans in cancer were the major significant KEGG enrichment pathways obtained from the analysis.

In order to obtain the interaction relationships of DEPs, the top 25 proteins with connectivity were selected and a protein-protein interaction (PPI) network diagram was drawn by searching the STRING database (https://string-db.org). The first 25 nodes with

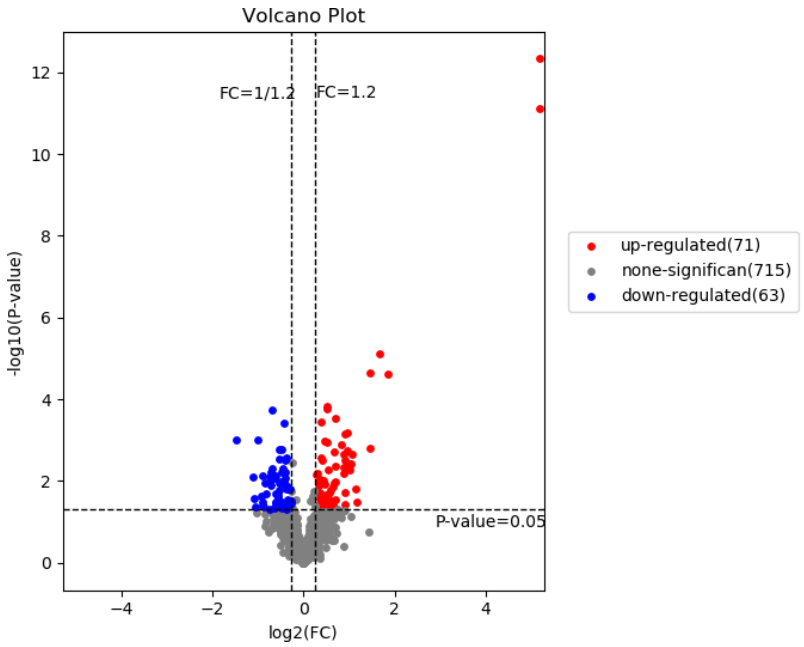


FIGURE 1
A volcano plot of the differentially expressed proteins. The red and blue dots represent significantly up-regulated and down-regulated DEPs, respectively. The horizontal dotted line represents p value <0.05, two vertical dashed lines indicate Fold change=1.2 and Fold change=0.83.

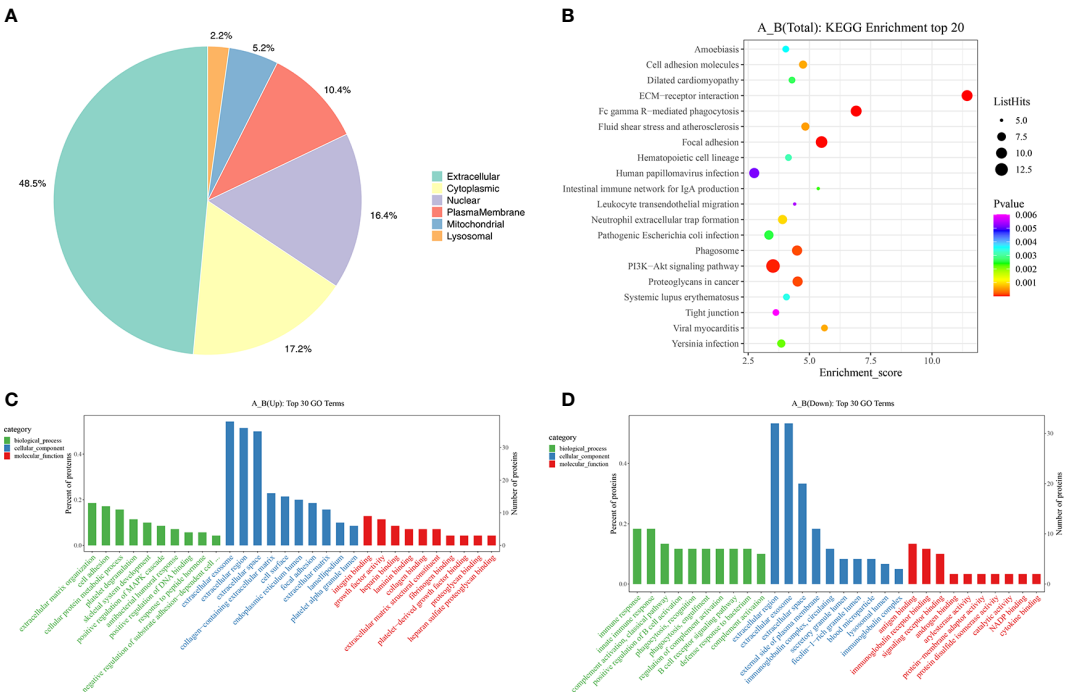


FIGURE 2
Subcellular localization and functional enrichment analysis of differentially expressed proteins. **(A)** Subcellular localization of the differentially expressed proteins. **(C)** GO terms of the differentially expressed proteins in the up-regulated group. **(D)** GO terms of the differentially expressed proteins in the down-regulated group. **(B)** The top 20 bubbles of KEGG enrichment in the differentially expressed proteins.

connectivity were visualized by using the Python package "network" and these are shown as protein IDs (Figure 3). Among these, only 3 proteins were down-regulated, and the remaining 22 proteins were up-regulated. Then, the network topology was analyzed, and the 25 proteins were displayed according to their degree of involvement as shown in Table 2.

## Metabolomic analysis

Figure 4 shows the PCA of all the samples, including the QCs. The QC samples were closely clustered in the middle of all the samples indicating that the analytical equipment used was stable and the experimental data was reliable. In the OPLS-DA, the intercepts of R2 and Q2 were 0.431 and -0.434, respectively, after 200 displacement tests. As shown in Figure 5A, the Q2 value was less than zero and there was no over-fitting of the model, which indicates that the model was reliable and effective. The OPLS-DA scores of the two groups showed a significant difference (Figure 5B). 103 significantly DEMs were found, of which 42 were up-regulated and 61 were down-regulated in the CPP group when compared to the normal group (>1-fold or <1-fold). All the 103 DEMs can be visualized *via* a volcano plot as shown in Figure 6. Metabolic pathway enrichment analysis of the DEMs was performed based on KEGG database. The top 20 metabolic pathways are shown in Figure 7.

## Bioinformatic analysis-integrated analysis of proteomics and metabolomics

In our study, 10 girls with CPP and 10 age-matched healthy controls underwent simultaneous proteomic and metabolomic analysis, and the 10 pairs of data obtained were analyzed jointly. The top 20 relative content data of DEPs and DEMs were extracted based on the p-values, and the correlation between proteins and metabolites was calculated by Pearson correlation analysis and a correlation heatmap was drawn (Figure 8). The differential proteins and metabolites were simultaneously mapped to the KEGG database in order to obtain any common pathways and 6 were obtained (Table 3).

The KGML database is a sub-library of the KEGG database, which contains both the relationships of graph objects in the KEGG pathway and information regarding the lineal homologous genes in the KEGG genes database. Using this information, a network relationship between proteins and metabolites can be

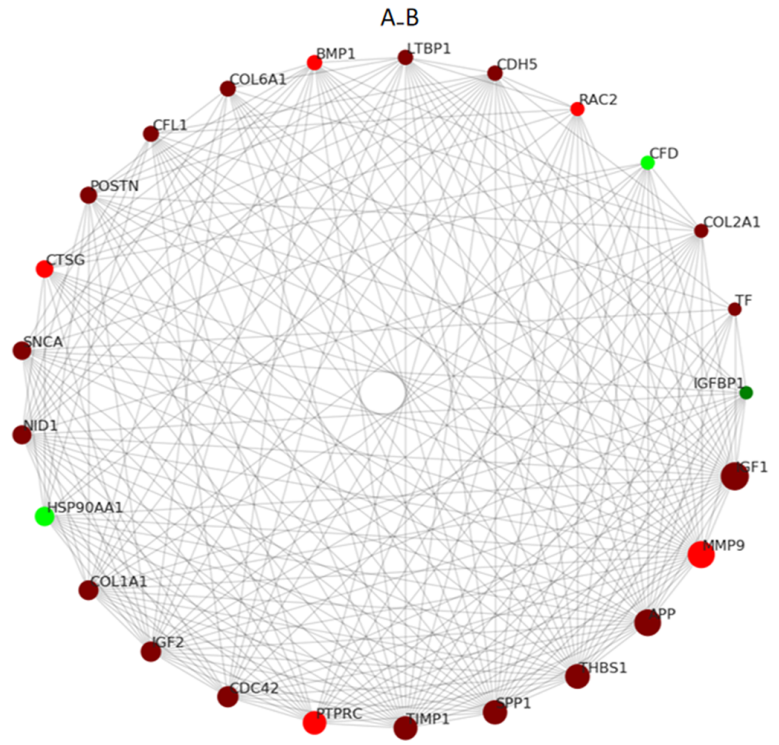


FIGURE 3
PPI network of the differentially expressed proteins in CPP. The circles in the figure indicate differentially expressed proteins, red circles represent up-regulated proteins and green circles represents down-regulated proteins. The size of the circle represents the degree of connection, and the larger the circle, the more connected it is.

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TABLE 2 A list of topological information of the network nodes involved.

| Accession | Gene Name | FC | Degree |
|---|---|---|---|
| P05019 | IGF1 | 1.567642216 | 62 |
| P14780 | MMP9 | 2.071147156 | 59 |
| P05067 | APP | 1.402941913 | 58 |
| P07996 | THBS1 | 1.6143275 | 52 |
| P10451 | SPP1 | 1.388791725 | 51 |
| P01033 | TIMP1 | 1.548383073 | 50 |
| P08575 | PTPRC | 1.876677053 | 49 |
| P60953 | CDC42 | 1.602653542 | 43 |
| P01344 | IGF2 | 1.343936209 | 41 |
| P02452 | COL1A1 | 1.623211919 | 40 |
| P07900 | HSP90AA1 | 0.729889712 | 39 |
| P14543 | NID1 | 1.255191999 | 38 |
| P37840 | SNCA | 1.579364545 | 37 |
| P08311 | CTSG | 2.095382839 | 35 |
| Q15063 | POSTN | 1.386956741 | 34 |
| P23528 | CFL1 | 1.480656624 | 32 |
| P12109 | COL6A1 | 1.228885387 | 32 |
| P13497 | BMP1 | 2.225164023 | 31 |
| Q14766 | LTBP1 | 1.442962676 | 31 |
| P33151 | CDH5 | 1.228331459 | 31 |
| P15153 | RAC2 | 1.862280196 | 29 |
| P02458 | COL2A1 | 1.283357367 | 29 |
| P00746 | CFD | 0.754562337 | 29 |
| P02787 | TF | 1.239288 | 28 |
| P08833 | IGFBP1 | 0.531224263 | 28 |

obtained, which is a convenient way to study the interactions between the proteome and metabolome in a systematic way, as shown in Figure 9. PC (16:1(9Z)/16:1(9Z)) was up-regulated in four of the metabolic pathways, including those involved in arachidonic acid, glycerophospholipid, linoleic acid and alpha-linolenic acid metabolism. The protein, P17174 (aspartate aminotransferase encoded by gene *GOT1*), was down-regulated in seven metabolic pathways involving the amino acids phenylalanine, tyrosine, tryptophan, arginine, alanine, aspartic and glutamic acids, cysteine, methionine, arginine and proline.

## Discussion

To the best of our knowledge, our study is the first to integrate proteomics and metabolomics to analyze serum samples obtained from girls with CPP. Using proteomic analysis, it was found that several different proteins in girls with CPP were mainly distributed in the extracellular region (48.5%) when compared with normal girls, suggesting that many of the active proteins played roles in these parts of the cells. MF analysis showed that the main functions associated with the differential proteins were related to their binding abilities. These results were consistent with a previous proteomic study of girls with CPP (24). The up-regulated proteins were mainly enriched in extracellular matrix organization, cell adhesion and cellular protein metabolic process, platelet degranulation and skeletal system development, which were related to pubertal growth and development. The down-regulated proteins were

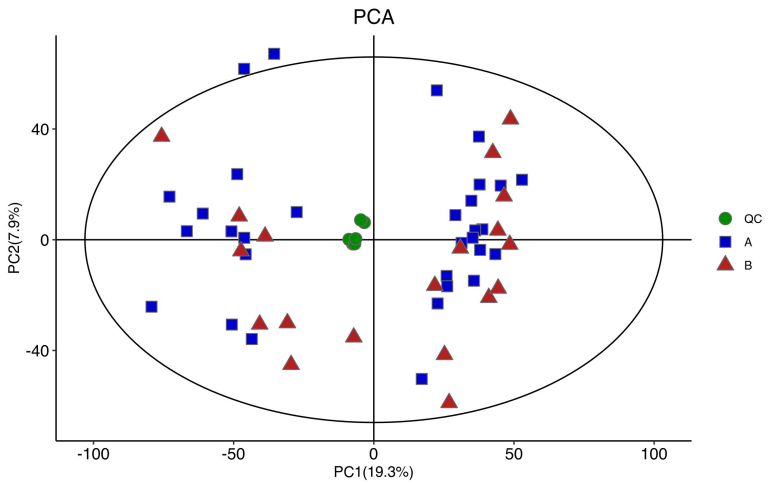


FIGURE 4
A PCA diagram of the samples used for analysis (including QC). QC samples were closely clustered in the middle of all samples, and no outlier samples were found, indicating good stability of instrumental analysis system and stable and reliable experimental data.

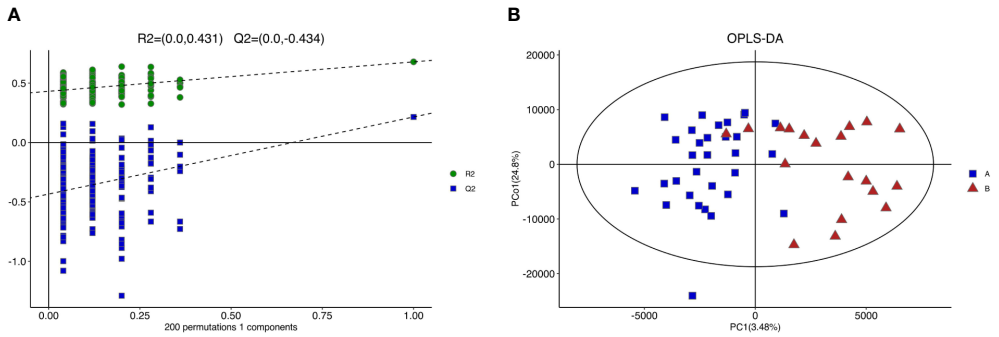


FIGURE 5
Results of replacement test and OPLS-DA score of girls with CPP vs the control group. **(A)** The closer R2Y was to 1, the more stable and effective the model was. Q2<0 indicated that the model was reliable and effective without over-fitting. **(B)** There was significant difference in OPLS-DA score between the two groups.

mainly enriched in immune response which might be involved in the development of CPP by participating in the regulation of sex hormones during puberty (25, 26).

In order to find the core proteins that are linked to CPP, the top 25 proteins with connectivity were selected to construct a PPI network and IGF-1 was found to have the highest connectivity. IGF-1 has been previously shown to have a predictive role as a biomarker in the diagnosis of girls with CPP (27). IGF2 and IGFBP1 were also thought to be involved in the process of puberty (28, 29). In addition, this study revealed other new candidate proteins such as MMP9, TIMP1, SPP1, CDC42, POSTN, COL1A1, COL6A1, COL2A1 and BMP1. Among them, MMP9, TIMP1, SPP1, CDC42, COL1A1, COL2A1, COL6A1 and BMP1 were previously correlated with the skeletal development and maturation (30–35). In addition, MMP9, TIMP1, SPP1 and POSTN were shown to play a role in regulating fat metabolism and insulin resistance (30, 36–38). MMP9, TIMP1, CDC42 and BMP1 were also involved in follicular development

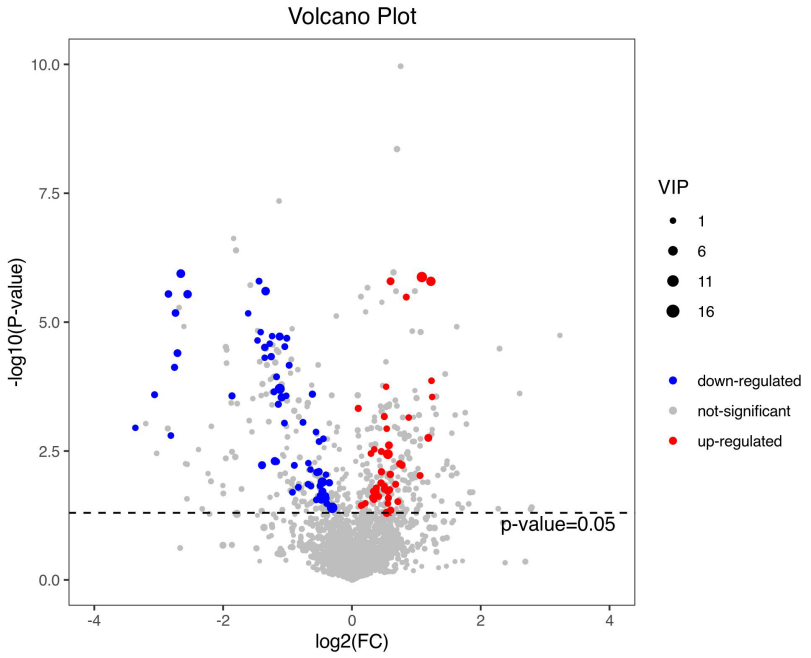


FIGURE 6
A volcano plot of the differential metabolites. The volcano map can be used to visualize p value, VIP value and FC value, which is beneficial to screen differential metabolites. The red and blue dots represent significantly up-regulated and down-regulated DEMs, respectively.

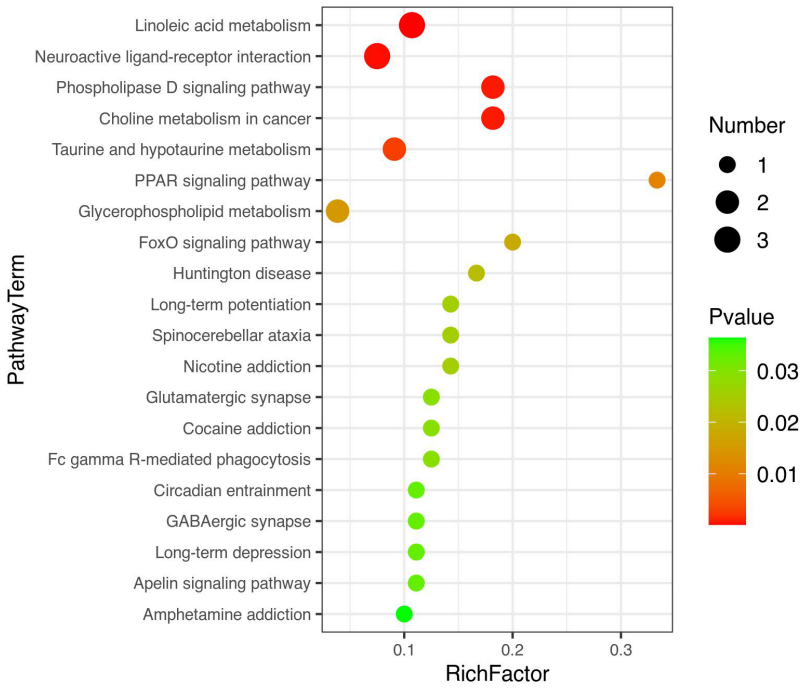


FIGURE 7
A bubble diagram of top-20 metabolic pathways. The color from green to red indicates that p-values decrease successively. The larger the point is, the more metabolites are enriched into the metabolic pathway.

(39–43), suggesting that these proteins may be closely related to pubertal development. However, these proteins had not been previously reported to be related to CPP, and their mechanisms needs to be further studied.

Subsequently, a metabolomic analysis was performed and 103 DEMs were identified, which were mainly enriched in linoleic acid metabolism, neuroactive ligand receptor interaction, phospholipase D signaling pathway, choline metabolism in cancer, taurine and low taurine metabolism pathways. In the linoleic acid metabolic pathway, the serum levels of linoleic acid and its metabolic derivatives (9-HODE and 12,13-DHOME) in CPP girls were significantly higher than those found in the normal group.

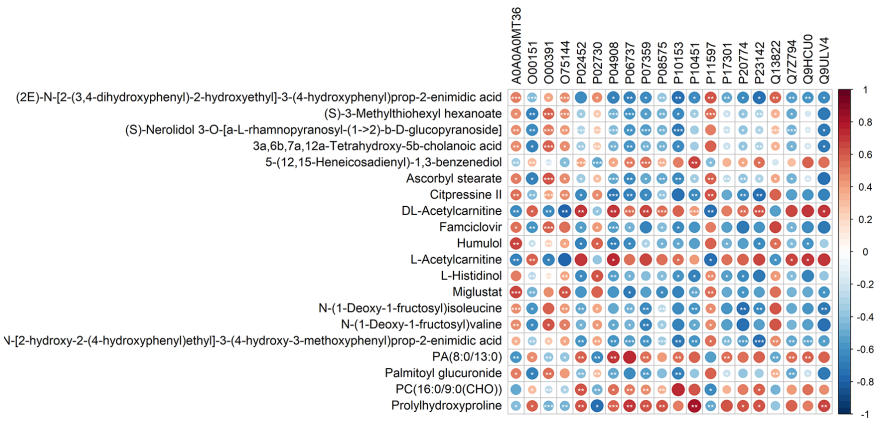


FIGURE 8
Correlation analysis of the differential proteins and metabolites. In the figure, red is positively correlated and blue is negatively correlated. *** represents correlation p value less than 0.001, ** represents correlation p value less than 0.01, and * represents correlation p value less than 0.05.

TABLE 3 Six pathways showing the differential proteins and metabolites involved.

| Pathway | Pathway name | Gene | Metabolite |
|---|---|---|---|
| Hsa04931 | Insulin resistance | PYGL | L-Acetylcarnitine<br>DL-Acetylcarnitine |
| Hsa05231 | Choline metabolism in cancer | RAC2 | PC (16:1(9Z)/16:1(9Z))<br>LysoPC (17:0)<br>Glycerophosphocholine |
| Hsa00270 | Cysteine and methionine metabolism | GOT1 | Phosphohydroxypyruvic acid |
| Hsa00400 | Phenylalanine, tyrosine and tryptophan biosynthesis | GOT1 | 3α,6β,7α,12α-Tetrahydroxy-5β-cholanoic acid |
| Hsa00590 | Arachidonic acid metabolism | GPX3 | PC (16:1(9Z)/16:1(9Z)) |
| Hsa00565 | Ether lipid metabolism | ENPP2<br>PLA2G7 | Glycerophosphocholine |

Lineolic acid is a polyunsaturated and is an essential fatty acid in humans where it stimulates insulin secretion (44). However, excessive long-term amounts of this fatty acid in the diet can aggravate a metabolic response resulting in an intestinal microflora imbalance in obese diabetic rats (45). Linoleic acid and its metabolic derivatives may be involved in CPP by regulating glucose and lipid metabolism.

In the neuroactive ligand receptor interaction pathway, sphingosine-1-phosphate (S1P), taurine and glutamate play important roles in neurotransmission and energy metabolism. The S1P/S1PR1/ceramide axis can activate the hunger signaling pathway in the hypothalamus through S1P so as to increase energy consumption and lipolysis, reduce food intake, thereby producing anti-obesity effects (46). Taurine is a sulfur amino acid that can

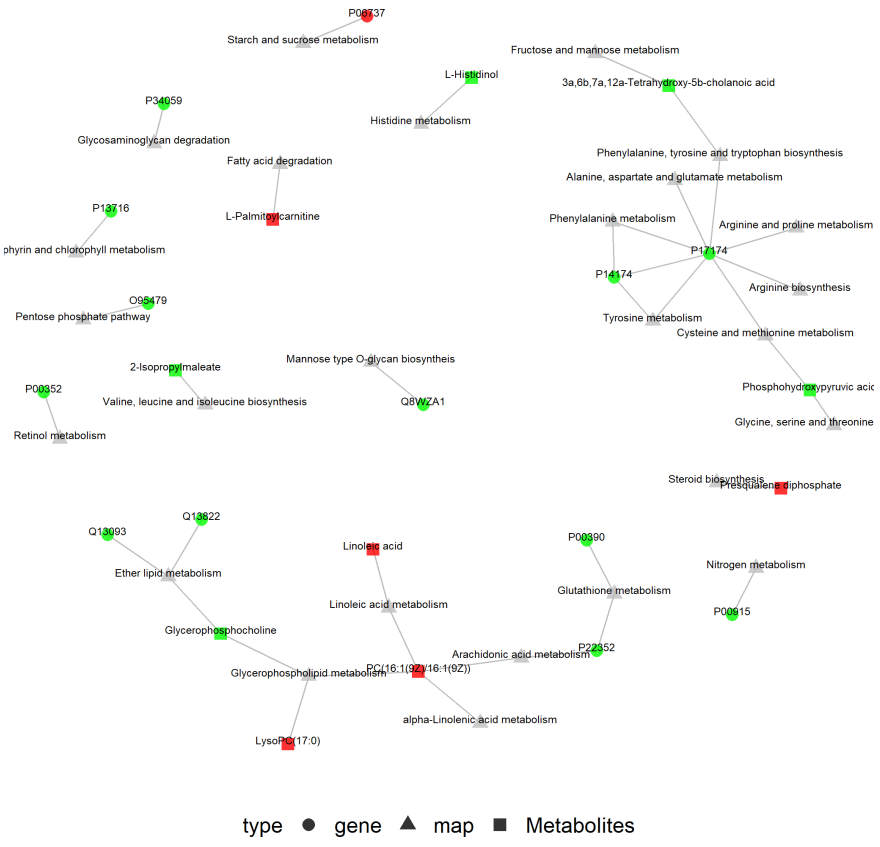


FIGURE 9
KGML network of the differential genes and metabolites. The squares represent genes and the triangles represent metabolites. Red represents up-regulated genes or metabolites, and bright green represents down-regulated genes or metabolites.

induce browning of white adipose tissue, resulting in increased energy expenditure and adaptive thermogenesis (47). Taurine also has an anti-obesity effect through a combination of actions, including stimulating energy expenditure, improving lipid metabolism, suppressing appetite and inhibiting oxidative stress (48). In our study, the serum S1P and taurine levels of CPP girls were higher than those of the normal group, indicating that girls with CPP had enhanced energy metabolism and were in need extra energy. In addition, previous studies have shown that neuroactive ligand receptors in the neuroactive ligand receptor interaction pathway such as KISS1/KISS1R, TAC3/TACR3, NPY/NPYR and LEP/LEPR can play a role in the occurrence and development of CPP. Yang (49) conducted a metabonomic study using urine samples obtained from girls with CPP and found that several metabolites in their urine were correlated more with the activity of the nervous system rather than the endocrine system. A variety of neuroactive ligands and their receptors may act together in the CNS thereby promoting the occurrence and development of CPP. The neuroactive ligand receptor interaction may be an important metabolic pathway in CPP.

Finally, an integrated bioinformatic analysis of proteomics and metabolomics was carried out. KGML network analysis showed that PC (16:1(9Z)/16:1(9Z)) was involved in the metabolism of arachidonic acid, glycerophospholipid and linoleic and α-linolenic acids. PC (16:1(9Z)/16:1(9Z)) is a phosphatidylcholine and it acts on LysoPC (17:0) as well as arachidonic, linoleic and α-linolenic acids in four metabolic pathways. Excessive phosphatidylcholine would generate a large amount of arachidonic acid through the linoleic and arachidonic acid metabolic pathways. Increased arachidonic acid production would, in turn, generate excessive prostaglandins through the arachidonic acid metabolic pathways. Prostaglandins could further stimulate the release of LHRH, which would stimulate gonadotropin secretion and interact with sex hormones to affect follicular development and ovulation (50), thus playing an important role in the pathogenesis of CPP. Arachidonic acid, glycerophospholipid and linoleic acid metabolism may be important metabolic pathways involved in the development of puberty in girls. In addition, PC (16:1(9Z)/16:1 (9Z)) may be a potentially important biomarker for CPP. However, there were some limitations to our study: 1. pubertal healthy girls were not included as controls in this study; 2. the sample size studied was small, and further a large sample of cohorts is needed to verify some of our findings; 3. the BMI, diet and other general living characteristics of the subjects were not controlled in this study, which might have caused some interference to the results obtained.

In conclusion, we conducted proteomics and metabolomics of serum samples of girls with CPP and found that they presented important differential proteins, metabolites and key metabolic pathways, which could provide novel ideas and clues to explore the pathogenesis of CPP. Subsequent PPI and KGML network analysis, yielded core proteins and metabolites that may be closely related to pubertal development and may be used as serum biomarkers for CPP. Arachidonic acid, glycerophospholipid and linoleic acid metabolism may also be additional metabolic pathways involved in the development of puberty in girls. However, more animal and cell experiments are needed to further clarify the role of these metabolic pathways in the pathogenesis of CPP.

## Data availability statement

Supplementary datasets to this article are available online - DOI for Figshare data: 10.6084/m9.figshare.20206184 (https://doi.org/10.6084/m9.figshare.20206184). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) *via* the iProX partner repository (51) with the dataset identifier PXD035132.

## Ethics statement

The studies involving human participants were reviewed and approved by Scientific Ethics Committee of The First Affiliated Hospital of Guangxi Medical University in Nanning, China (2022 (KY-E-025)). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## Author contributions

DL and ML participated in study design and drafted the manuscript. ML and YC collected the cases and performed the laboratory assays. The final manuscript was approved by all the authors.

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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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# Pubertal timing in children with Silver Russell syndrome compared to those born small for gestational age

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**Context:** Data on pubertal timing in Silver Russell syndrome (SRS) are limited.

**Design and methods:** Retrospective observational study including twenty-three SRS patients [11p15 loss of methylation, (11p15 LOM, n=10) and maternal uniparental disomy of chromosome 7 (mUPD7, n=13)] and 21 small for gestational age (SGA). Clinical (thelarche in females; testis volume ≥ 4 ml in males; pubarche), BMI SD trend from the age of 5 to 9 years to the time of puberty, biochemical parameters of puberty onset [Luteinizing hormone (LH), 17-β-estradiol, testosterone], and bone age progression were evaluated

**Results:** Pubertal onset and pubarche occurred significantly earlier in children with SRS than in SGA (p 0.03 and p 0.001, respectively) and clinical signs of puberty onset occurred earlier in mUPD7 than in 11p15LOM group (p 0.003). Five SRS children experienced central precocious puberty and LH, 17-β-estradiol, testosterone were detected earlier in SRS than in SGA (p 0.01; p 0.0001). Bone age delay in SRS children was followed by rapid advancement; the delta between bone age and chronological age in SRS group became significantly higher than in SGA group at the age of 9-11 years (p 0.007). 11p15LOM patients were underweight at the age of 5 years and showed a progressive normalization of BMI that was significantly higher than in mUPD7 (p 0.04) and SGA groups (p 0.03) at puberty onset.

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**Conclusion:** Timing of puberty is affected in SRS and occurred earlier in mUPD7 compared to 11p15LOM. The impact of early puberty on adult height and metabolic status deserves long-term evaluation.

KEYWORDS

puberty, bone age, silver russell syndrome, 11p15 LOM, mUPD7

## Introduction

Silver-Russell syndrome (SRS) is an epigenetic disorder characterized by severe intrauterine (IUGR) and postnatal growth retardation with typical dysmorphic features and has an incidence between 1 in 70.000 and 1 in 100.000 live births (1). The most common reported genetic abnormalities are 11p15 ICR1 loss of methylation, (11p15 LOM) and maternal uniparental disomy of chromosome 7 (mUPD7), which occur in 30-60% and 5-10% of cases, respectively (2); single cases carry other chromosome abnormalities (3–5), including duplications of maternal 11p15 (6, 7). In a significant proportion of patients (40%), the molecular etiology remains unknown, and SRS remains primarily a clinical diagnosis, according to the Netchine-Harbison (NH) scoring system and the international consensus statement (2, 8). Indeed, a target next-generation sequencing approach in patients referred for SRS testing increases the mutation rate as well as other diagnoses overlapping SRS (4, 9–11)

Data on the natural history of puberty and bone age progression in patients with SRS are limited.

It is known that children who are born small for gestational age (SGA), including those with SRS, tend to have earlier and rapidly progressing puberty, with faster bone maturation and a shorter period of pubertal peak height velocity, associated with metabolic abnormalities such as visceral adiposity (12–15). According to the available literature, onset of puberty in SRS is usually within the normal range (8–13 years in girls and 9–14 years in boys) but at the younger end of the spectrum (2, 12, 15, 16). SRS children (particularly those with 11p15LOM) can experience an early and rapidly progressive adrenarche in comparison with non-SRS SGA children (2, 12, 16, 17). In a retrospective study including 62 subjects with clinical diagnosis of SRS, Binder et al. showed that premature adrenarche was more frequent in SRS than in general population and was associated with early age at initiation of GH treatment. However, growth response to GH treatment and adult height were not compromised by early adrenarche in this cohort (17).

In SRS patients with early adrenarche, the onset of central puberty might be earlier and the tempo faster than expected. In the past few decades, population studies analyzing the timing of normal puberty observed a mean age of puberty onset of 9.7–10 years in healthy girls (2, 15), with an earlier thelarche in girls born more recently (mean age 9.86 years in European girls born in 2006 versus 10.88 years in those born in 1991) (18). One hypothesis to explain the trend towards younger age of pubertal onset in girls is that an earlier maturation can be the expression of an adaptative mechanism to escape from ectopic adiposity which, in turn, ensues from a mismatch between reduced prenatal weight gain and increased postnatal weight gain (19, 20).

SRS girls seem to start activation of hypothalamic-pituitary-gonadal axis at a mean age of 9.1 years (2) This early puberty further accelerates bone age maturation, which leads to an attenuated pubertal growth spurt and compromised adult height (2). Children with mUPD7 are likely to progress to central puberty at an even younger age than patients with SRS and 11p15 LOM (mean starting age 8.5 years in girls and 9.5 years in boys) (2). A rapid increase in body mass index (BMI) might also exacerbate the tendency to early adrenarche and central puberty (2, 15, 16).

In this study, we investigated the clinical and biochemical features of puberty onset and the evolution of bone age and BMI over time in a cohort of genetically confirmed SRS patients compared to subjects born SGA.

## Patients and methods

This is a retrospective observational study, including patients with a confirmed diagnosis of SRS recruited at the Pediatric Endocrine Unit, Istituto Giannina Gaslini, University of Genova (Genova, Italy), between November 2014 and October 2021

Population includes:

- Patients with molecular diagnosis of SRS and with an age ≥ 5 years.
- Children born SGA who were 5 years old or older at the time of enrollment; SGA subjects were rigorously

selected in order to exclude chromosomopathies and syndromes; dysmorphic features, psychomotor delay, macrocephaly, microcephaly and comorbidities were exclusion criteria and a normal karyotype was mandatory.

The study was approved by the Institutional Review Board and it was approved by the ethical committee of Giannina Gaslini Institute (PRIN 2015. Number: 2015JHLY35). Written informed consent was obtained from the parents or legal guardians of all subjects according to the Declaration of Helsinki.

## Patients

The study group comprised 23 SRS subjects (11p15LOM n=10; mUPD7 n=13; 12 males; 11 females) and 21 SGA subjects (14 males; 7 females). Five SRS (3 11p15LOM; 2 mUPD7) patients (22%) required nasogastric tube feeding for the first weeks of life. Growth hormone (GH) treatment was undertaken in 18 of 23 (78%) SRS children and in 13 of 21 (61%) SGA children and the mean duration of GH treatment was 6.9 ± 3 years in SRS and 6.9± 2.9 years in SGA; the age at the start of treatment was 4.7± 2.6 years in SRS and 7.8 ± 2.6 years in SGA; the GH dose was between 0.034 and 0.035 mg/kg/day in both groups. 3 subjects with SRS and 4 with SGA reached the adult height.

## Genetic analysis

Genomic DNA was extracted from peripheral white blood cells using the Wizard® Genomic DNA Purification Kit (Promega). Patients analyzed by molecular testing before 2008: from parents to proband segregation by microsatellite analysis spanning the whole chromosome 7 was performed to disclose mUPD7. Patients addressed to molecular testing after 2008: hypomethylation at H19/IGF2: IG-DMR was first analyzed by Southern-Blot hybridization of H19-DMR or by MS-MLPA kit ME030-C3 BWS/RSS (MRC-Holland, Amsterdam, The Netherlands). Patients with a balanced methylation pattern at H19/IGF2: IG-DMR underwent to mUPD7 analysis.

## H19/IGF2: IG-DMR methylation analysis

Genomic DNA was digested with Csp6I/HpaII and BamHI/NotI restriction enzymes, respectively, before southern blot hybridization to H19-DMR (provided by Prof. A. Riccio, CNR Institute of Genetics and Biophysics, Naples). MS-MLPA analysis was carried on cases and control samples according to the kit instructions and analyzed by Coffalyser.net software. The protocols and quantitative analysis to detect low level of mosaicism and aberrant copy number were previously described

## mUPD7 Analysis

A standard panel of microsatellite markers D7S517 (7p22.2), D7S513 (7p21.3), D7S507(7p21.1), D7S503 (7p21.1), D7S2493 (7p15.3), D7S2525 (7p15.2),D7S2496 (7p14.3), D7S519 (7p13), D7S2422 (7p12.1), D7S2467(7p12.1), D7S506(7p12.1), D7S1870 (7q11.23), D7S669 (7q21.1), D7S486 (7q31.2), D7S640(7q32.3), D7S798 (7q36.2), D7S2465(7q36.3) was investigated. In case of not informative markers, additional closely mapped microsatellites were analysed. PCR fragments were separated by capillary electrophoresis on the automated ABI 310 sequencer and data analysed using the Genemapper software (Applied Biosystem).

## Auxological and pubertal data

Data on gestational age as well as birth weight, birth length and head circumference were obtained from birth charts and converted to SD according to Bertino et al. (21).

Auxological data were evaluated according to Tanner growth charts (22). BMI was calculated according to Tanner growth charts and the trend was evaluated at different ages (from the age of 5 to the age of 9 years) and at the age of clinical puberty onset. In addition, weight, length and BMI data as well as weight gain from birth to 4 years and delta BMI gain from age 2 to 4 years (with a variability of ± 2 months) were collected from growth charts when available. Clinical pubertal onset was defined as testicular volume ≥ 4 ml in boys and breast development (B2 Tanner stage) in females (23).

Bone age was assessed by Greulich and Pyle method (24) and the bone age progression was evaluated from the age of 5 to the age of 11 years by the same radiologist and by the same pediatric endocrinologist (GP). Hormonal parameters [Luteinizing hormone (LH), 17-β-estradiol, testosterone] were evaluated by chemiluminescent assay (Roche). LH, 17-β-estradiol and testosterone were considered detectable if ≥0.1 U/L, ≥ 5 pg/ml, ≥5 ng/dl, respectively.

## Statistical analysis

Data are described as mean and standard deviation (SD) or median and range for continuous variables, and as absolute and relative frequencies for categorical variables.

Non parametric analysis (Mann-Whitney U-test), for continuous variables and the Chi square or Fisher's exact test for categorical variables were used to measure differences between groups. Statistical analysis was performed using SPSS for Windows (SPSS Inc, Chicago, Illinois USA). Pairwise correlation analysis between anthropometrics data at birth,

early feeding characteristics, BMI trend over time and clinical and biochemical pubertal onset data was performed; p values ≤ 0.05 were considered statistically significant, and all p values were based on two-tailed tests.

# Results

## Patient's characteristics

The mean age at the last evaluation was 11.3 ± 3.9 SD years in SRS group and 14.2 ± 1.9 SD years in SGA group.

**Birth**. Two subjects with 11p15LOM were born by *in vitro* fertilization and Intra-Cytoplasmic Sperm Injection, respectively. The mean gestational age was 36.4 ± 2.3 weeks in SRS and 39.2± 1.5 weeks in SGA.

All 11p15LOM patients were born SGA versus 76% in mUPD7 group. Birth length SD was shorter in 11p15LOM group than in mUPD7 group (p 0.06) and SGA group (p 0.05) (Table 1). Birth weight (grams) was lower in SRS than in SGA (p 0.0001), although there was no significant difference in terms of birth weight SD; head circumference SD was greater in SRS than in SGA (p 0.02). Birth data and auxological data are reported in Table 1.

**Pubertal onset.** The mean age at the last evaluation was 11.3 ± 3.9 SD years in SRS group and 14.2 ± 1.9 SD years in SGA group. Clinical pubertal onset and pubarche occurred significantly earlier in SRS group than in SGA group (p 0.03 and p 0.001, respectively) and serum LH and sexual hormones became detectable earlier in SRS than in SGA (p 0.01; p 0.0001), (Table 2).

mUPD7 patients presented a significantly earlier pubertal onset compared to11p15LOM subjects (p 0.003). The age of pubarche as well as the age at which sexual hormones became detectable in serum was earlier in mUPD7 if compared to 11p15 LOM group, although not significantly (p 0.16 and p 0.27, respectively) (Table 2). Five SRS children, 21.7% of the group, (3 females and 2 males, 2 11p15LOM and 3 mUPD7) experienced central precocious puberty; 4 out of 5 were treated with gonadotropin-releasing hormone (GnRH) analog; 1 girl who came to our attention at the age of 15 years had experienced menarche at the age of 10 years while the two boys (1 with 11p15LOM; 1 with mUPD7) showed a biochemical central precocious pubertal activation but with small testis (testicular volume 3 ml in both subjects).

**BMI evolution and feeding problems.** Patients with 11p15LOM were underweight at the age of 5 years, then showed a progressive normalization of BMI over time and a significantly higher BMI SD at the age of puberty onset compared to mUPD7 group (p 0.04) and SGA group (p 0.03) (Table 3), (Figure 1).

Similarly, the gap between bone age and chronological age (negative at the age of 5-7 years) in SRS group became significantly higher than in SGA group at the age of 9-11 years (p 0.007) (Table 3).

Although no significant correlation between nasogastric tube feeding and early pubertal onset was found because of the small number of patients, it should be considered that 3 out of 5 tube-fed subjects experienced precocious puberty (60%). Available data of weight and length gain from birth to 4 years (with a variability of ± 2 months) are reported in Table 4.

# Discussion

To our knowledge, this is the first study that evaluated the clinical and biochemical characteristics of pubertal timing in association with the progression of bone age and BMI trends in genetically confirmed SRS children compared to children born SGA.

TABLE 1 Birth data and auxological data at last evaluation of SGA and SRS (divided into 11p15LOM and UPD7 groups) patients.

| | | | | | p-values | | | |
|---|---|---|---|---|---|---|---|---|
| | **SRS (23 pt)** | **11p15LOM (10 pt)** | **UPD7 (13 pt)** | **SGA (21 pt)** | **SRS vs SGA** | **11p15 vs UPD7** | **11p15LOM vs SGA** | **UPD7 vs SGA** |
| **Gestational age (w)** | 36.4 ± 2.3 | 36.64 ± 2.57 | 36.31 ± 2.10 | 39.2 ± 1.5 | **0.0001** | 0.98 | **0.002** | **0.0001** |
| **Birth weight (gr)** | 1823 ± 468 | 1697 ± 501 | 1919 ± 436 | 2415 ± 338 | **0.0001** | 0.28 | **0.001** | **0.001** |
| **Birth weight SD** | -2.29 ± 0.75 | -2.61 ± 0.75 | -2.04 ± 0.67 | -2.19 ± 0.42 | 0.93 | 0.06 | 0.12 | 0.25 |
| **Birth HC (cm)** | 31.93 ± 1.96 | 32.51 ± 2.13 | 31.50 ± 1.82 | 32.69 ± 1.28 | 0.48 | 0.13 | 0.80 | 0.18 |
| **Birth HC SD** | -0.78 ± 0.83 | -0.35 ± 0.77 | -1.08 ± 0.76 | -1.42 ± 0.44 | **0.02** | 0.11 | **0.002** | 0.21 |
| **Birth length** | 41.54 ± 3.71 | 40.50 ± 3.57 | 42.42 ± 3.75 | 45.92 ± 1.99 | **0.001** | 0.28 | **0.001** | **0.02** |
| **Birth length SD** | -2.54 ± 1.06 | -3.01 ± 0.98 | -2.15 ± 0.99 | -2.18 ± 0.77 | 0.34 | 0.06 | **0.05** | 0.82 |
| **Age at last evaluation (y)** | 11.3 ± 3.9 | 12.76 ± 4.50 | 10.12 ± 3.11 | 14.2 ± 1.9 | **0.002** | 0.17 | 0.27 | **0.0001** |
| **Target height SD** | 0.03 ± 0.80 | 0.10 ± 1.06 | -0.02 ± 0.61 | -0.76 ± 1.18 | 0.06 | 0.79 | 0.15 | 0.11 |
| **Δ from target height SD** | -1.43 ± 1.50 | -1.90 ± 1.79 | -1.10 ± 1.24 | -1.07 ± 1.15 | 0.53 | 0.43 | 0.35 | 0.89 |

w, weeks; y, years; SGA, small for gestational age; SRS, Silver-Russell syndrome; Δ, delta; gr, grams; HC, head circumference; 11p15 LOM, 11p15 loss of methylation; UPD7, maternal uniparental disomy of chromosome 7.
The bold numbers indicate a statically significant p-value ( p value ≤ 0.05).

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TABLE 2 Puberty onset characteristics in SRS and SGA groups.

| | | | | | p-values | | | |
|---|---|---|---|---|---|---|---|---|
| | SRS | 11p15LOM | UPD7 | SGA | SRS vs SGA | 11p15 vs UPD7 | 11p15 vs SGA | UPD7 vs SGA |
| **Clinical puberty onset (y)** | 10.02 ± 1.80 (13 pt) [7.0-13.0] | 11.75 ± 1.14 (5 pt) [10.0-13.0] | 8.94 ± 1.15 (8 pt) [7.0-10.0] | 11.38 ± 1.15 (18 pt) [8.0-13.5] | **0.03** | **0.003** | 0.53 | **0.0001** |
| **Clinical puberty onset (y) in males** | 11.46 ± 1.25 (6 pt) [8.0-13.0] | 12.13 (4 pt) [11.5-13.0] | 10.0 (2 pt) [10.0] | 11.79 ± 1.48 (12 pt) [8.0-13.5] | 0.61 | - | – | - |
| **Clinical puberty onset (y) in females** | 8.79 ± 1.15 (7 pt) [7.0-10.0] | 10.0 (1 pt) | 9.0 (6 pt) [7.0-10.0] | 10.57 ± 0.96 (6 pt) [9.25-12.0] | **0.02** | - | – | - |
| **Pubarche age (y)** | 9.92 ± 1.38 (14 pt) [7.5-11.5] | 10.34 ± 1.66 (7 pt) [7.5-11.5] | 9.49 ± 0.97 (7 pt) [8.0-10.5] | 11.90 ± 1.70 (18 pt) [8.0-14.5] | **0.001** | 0.16 | **0.04** | **0.002** |
| **Pubarche age (y) in males** | 10.33 ± 1.49 (8 pt) [7.5-11.5] | 11.5 (5 pt) [7.5-11.5] | 10.0 (3 pt) [8.8-10.4] | 12.37 ± 1.80 (12 pt) [8.0-14.5] | **0.01** | – | - | - |
| **Pubarche age (y) in females** | 9.37 ± 1.10 (6 pt) [8.0-10.5] | 9.50 (2 pt) [8.5-10.5] | 9.38 (4 pt) [8.0-10.5] | 10.97 ± 1.06 (6 pt) [9.8-12.5] | 0.09 | – | - | - |
| **Measurable sexual hormones*, age (y)** | 9.47 ± 1.44 (12 pt) [7.0-12.3] | 10.12 ± 1.61 (5 pt) [8.0-12.3] | 9.01 ± 1.22 (7 pt) [7.0-10.8] | 11.86 ± 1.28 (19 pt) [9.8-14.7] | **0.0001** | 0.27 | **0.02** | **0.0001** |
| **Measurable sexual hormones*, age (y) in males** | 9.92 ± 1.36 (8 pt) [8.0-12.3] | 9.82 (5 pt) [8.0-12.3] | 9.19 (3 pt) [8.8-10.8] | 12.09 ± 1.39 (13 pt) [9.9-14.7] | **0.01** | – | - | - |
| **Measurable sexual hormones*, age (y) in females** | 8.58 ± 1.30 (4 pt) [7.0-10.1] | 0 pt | 8.59 (4 pt) [7.0-10.1] | 11.38 ± 0.95 (6 pt) [9.8-12.7] | **0.03** | – | - | - |
| **Measurable LH**, age (y)** | 9.01 ± 1.53 (13 pt) [6.0-11.3] | 9.58 ± 2.07 (4 pt) [6.6-11.3] | 8.75 ± 1.29 (9 pt) [6.0-10.1] | 10.67 ± 1.75 (16 pt) [7.4-14.0] | **0.01** | 0.20 | 0.49 | **0.005** |
| **Measurable LH**, age (y) in males** | 9.47 ± 1.50 (7 pt) [6.6-11.3] | 10.04 (4 pt) [6.6-11.3] | 9.08 (3 pt) [8.8-9.8] | 10.9 ± 1.72 (12 pt) [8.0-14] | 0.1 | – | – | - |
| **Measurable LH**, age (y) in females** | 8.47 ± 1.51 (6 pt) [6.0-10.1] | 0 pt | 8.01 (6 pt) [6.0-10.1] | 9.99 ± 1.90 (4 pt) [7.4-11.9] | 0.3 | – | – | - |
| **Menarche age (y)** | 11.11 ± 0.69 (5 pt) [10.0-11.8] | 10.91 ± 1.29 (2 pt) [10.0-11.8] | 11.23 ± 0.25 (3 pt) [11.0-11.5] | 11.86 ± 0.75 (4 pt) [10.9-12.7] | 0.29 | 1 | 0.53 | 0.40 |

y, years; SRS, Silver-Russell syndrome; 11p15, 11p15 loss of methylation; UPD7, maternal uniparental disomy of chromosome 7; pt, patients; LH, luteinizing hormone * 17- β -estradiol ≥ 5 pg/ml; testosterone ≥ 5 ng/dl. ** LH ≥ 0.1 U/L.
- not applicable because of the little number of patients.
The bold numbers indicate a statically significant p-value ( p value ≤ 0.05).

The published literature on the natural history of puberty onset and bone age progression in patients with SRS is limited (2, 16, 17, 25, 26). In a retrospective study including 62 subjects with clinical diagnosis of SRS, Binder et al. showed that premature adrenarche was more frequent in SRS than in the general population and was associated with early age at initiation of GH treatment, but growth response to GH treatment and adult height were not compromised (17). The lack of a molecular diagnostic confirmation represents a significant limitation of this study preventing a comparison between molecular SRS groups (17). In 16 subjects with 11p15LOM, Canton et al. (16) showed that the age of onset of adrenarche was earlier than in general population and a marked increase in BMI was associated with premature adrenarche and early puberty. However, this study included only one molecular SRS group (11p15LOM) (16).

In a study comparing a cohort of SRS subjects (31 11p15 LOM, 11 mUPD7, 20 idiopathic SRS) and a cohort of patients born SGA non-SRS, puberty started significantly earlier in the former (at 10.2 years versus 11.2 years in girls with SRS and non-SRS SGA, respectively, and at 11.4 years versus 12.0 years in boys

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TABLE 3 BMI trend between 5 to 9 years related to bone age in SGA and SRS patients.

| | SRS (23 pt) | 11p15LOM (10 pt) | UPD7 (13 pt) | SGA (21 pt) | p-values | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | SRS vs SGA | 11p15 vs UPD7 | 11p15LOM vs SGA | UPD7 vs SGA |
| **BMI SD at 5y** | -2.07 ± 1.16 | -2.58 ± 1.43 | -1.80 ± 0.97 | -1.12 ± 0.98 | 0.90 | 0.33 | 0.09 | 0.18 |
| **BMI SD at 7y** | -1.48 ± 1.30 | -1.63 ± 1.48 | -1.39 ± 1.25 | -1.41 ± 1.01 | 0.98 | 0.96 | 1 | 0.98 |
| **BMI SD at 9y** | -0.72 ± 1.39 | -0.40 ± 0.75 | -0.89 ± 1.64 | -0.84 ± 1.46 | 0.85 | 0.28 | 0.35 | 0.70 |
| **BMI SD at puberty onset** | -0.31 ± 1.09 | 0.46 ± 0.83 | -0.80 ± 0.97 | -0.84 ± 1.20 | 0.20 | **0.04** | **0.03** | 0.85 |
| **Δ BMI from 5y to 9y** | 1.13 ± 1.02 | 2.3 ± 0.28 | 0.84 ± 0.91 | 0.03 ± 0.98 | **0.04** | 0.09 | 0.07 | 0.11 |
| **Δ BMI from 7y to 9y** | 0.75 ± 1.04 | 1.47 ± 1.25 | 0.48 ± 0.89 | 0.03 ± 0.36 | 0.06 | 0.08 | **0.007** | 0.31 |
| **Bone age-chronological age (at 5/7y)** | -1.71 ± 0.81 | -1.32 ± 0.86 | -1.92 ± 0.73 | -1.55 ± 0.95 | 0.79 | 0.26 | 0.61 | 0.46 |
| **Bone age-chronological age (at 7/9y)** | -1.03 ± 0.97 | -0.33 ± 0.78 | -1.37 ± 0.89 | -1.73 ± 1.24 | 0.15 | **0.05** | **0.04** | 0.46 |
| **Bone age-chronological age (at 9/11y)** | -0.18 ± 0.98 | -0.57 ± 1.23 | 0.16 ± 0.62 | -1.42 ± 1.25 | **0.007** | 0.14 | 0.38 | **0.001** |
| **Δ BA-CA from 5/7y to 9/11y** | 2.16 ± 0.86 | 1.7 ± 1.2 | 2.39 ± 0.65 | 0.43 ± 0.9 | **0.001** | 0.71 | 0.08 | **0.001** |
| **Δ BA-CA from 7/9y to 9/11y** | 1.38 ± 1.02 | 0.78 ± 0.68 | 1.63 ± 1.07 | 0.65 ± 0.52 | 0.08 | 0.38 | 0.78 | **0.04** |

BMI, body mass index; w, weeks; y, years; SGA, small for gestational age; SRS, Silver-Russell syndrome; 11p15, 11p15 loss of methylation; UPD7, maternal uniparental disomy of chromosome 7; BA, bone age; CA, chronological age; Δ, delta.
The bold numbers indicate a statically significant p-value ( p value ≤ 0.05).

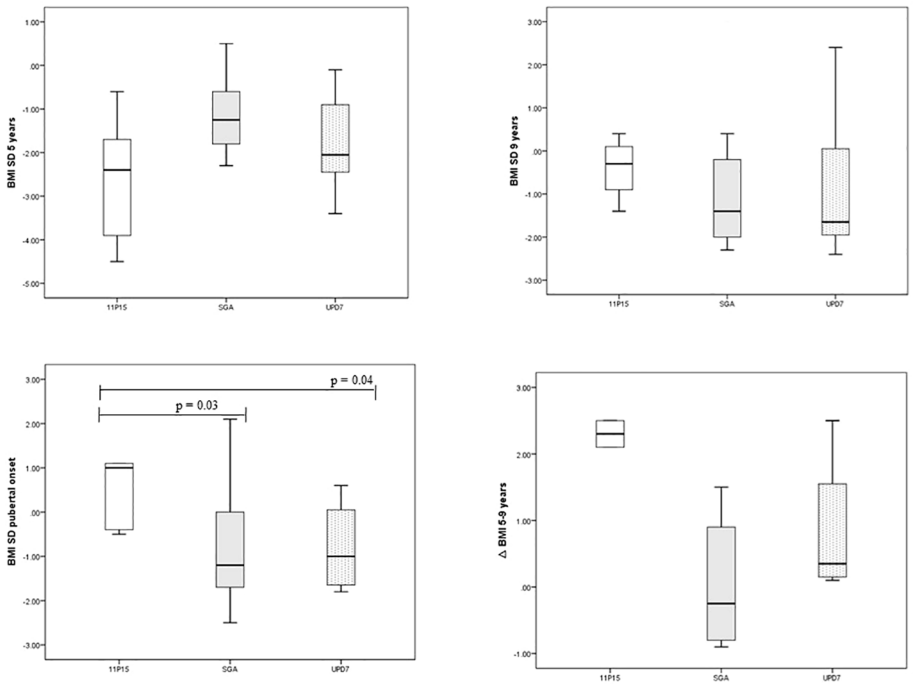


FIGURE 1
BMI trend in SRS and in SGA subjects.

with SRS and non-SRS SGA, respectively). In this study, boys with mUPD7 were the youngest at the onset of puberty and in 17 SRS patients puberty was postponed for 2 years with GnRH analogs due to a low predicted adult height (25). In a study including 31 SRS patients (15 11p15LOM, 7 mUPD7, 9 clinical diagnosis) and 123 non-SRS SGA subjects, Goedegebuure et al. showed a similar onset and progression of puberty in SRS and non-SRS SGA subjects (26).

Although there is little data on puberty onset in SRS, children born SGA are known to have an increased risk of

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TABLE 4 Available data of weight and length gain from birth to 4 years (variability ± 2 months) in SRS children and SGA subjects.

| | 11p15LOM (8pt) | UPD7 (11 pt) | SGA (7pt) | p value 11p15 vs UPD7 | 11p15 vs SGA | UPD7 vs SGA |
|---|---|---|---|---|---|---|
| **BMI SD at 2y** | -2.1 ± 1.5 | -3.07 ± 0.93 | -1.17 ± 0.54 | 0.34 | 0.34 | **0.001** |
| **Δ BMI SD from 2y to 3y** | -1.45 ± 2.7 | 0.53 ± 0.64 | | **0.04** | | |
| **Δ BMI SD from 2y to 4y** | -1.3 ± 3 | 0.7 ± 0.6 | | 0.06 | | |
| **Weight gain (kg) birth-1 year** | 4.1 ± 0.93 | 3.3 ± 0.56 | | 0.23 | | |
| **Weight gain (kg) Birth-4 years** | 8.63 ± 1.3 | 8.1 ± 1.44 | 9.6 ± 1.07 | 0.33 | 0.16 | **0.03** |
| **Weight gain (kg) 2-4 years** | 2.5 ± 1.6 | 3.1 ± 1.1 | | 0.66 | | |
| **Length gain Birth-1 year** | 23 ± 8.2 | 22.1 ± 4.5 | | 0.5 | | |
| **Height gain birth-4years** | 49.5 ± 6.6 | 44.9 ± 4.9 | 47.3 ± 4.2 | 0.07 | 0.34 | 0.37 |
| **Height gain (cm) 2-4 years** | 16.7 ± 3.6 | 15 ± 3.3 | | 0.34 | | |

BMI, body mass index; w, weeks; y, years; SGA, small for gestational age; SRS, Silver-Russell syndrome; 11p15, 11p15 loss of methylation; UPD7, maternal uniparental disomy of chromosome 7.
The bold numbers indicate a statically significant p-value ( p value ≤ 0.05).

developing early and rapidly progressing puberty, compromising thereby adult height and metabolic status (12, 13, 27–29). Nevertheless, limited data are available so far on therapeutic options in SGA children, including SRS, with a poor adult height expectation, with the exception of few studies on the use of GnRH analogs in combination with GH (12, 30–33). A double-blind clinical trial is still ongoing to investigate the efficacy of Anastrozole, a third-generation aromatase inhibitor, in slowing bone maturation in SRS (34).

It should be considered that a secular trend towards an earlier puberty onset in healthy children was observed in many countries. One hypothesis to explain the trend towards younger age of pubertal onset in girls is that an earlier maturation can be the expression of an adaptative mechanism to escape from ectopic adiposity which, in turn, ensues from a mismatch between reduced prenatal weight gain and increased postnatal weight gain (19, 20). Yanhui et al. have also recently showed an association between prepubertal adiposity and earlier puberty onset both in females and in males (35).

Since most SRS subjects are born SGA, the aim of our study was to evaluate the clinical and biochemical puberty characteristics in SRS compared to non-SRS SGA subjects and to evaluate the impact of BMI on puberty onset. In our cohort, puberty onset and pubarche occurred significantly earlier in SRS group than in SGA group and LH and sexual hormones were detected earlier in SRS than in SGA. In particular, within the SRS group, clinical signs of pubertal onset occurred earlier in mUPD7 than in 11p15LOM subjects. In addition, according to the literature, our SRS group showed a delay of bone age followed by rapid acceleration at the age of 9-11 years (2).

In agreement with Canton et al. (16), we found that the BMI of SRS children progressively increases with age. Notably, patients with 11p15LOM who were underweight at the age of 5 years showed a progressive normalization of BMI which was significantly higher at the time of onset of puberty compared to BMI of mUPD7 and SGA groups. However, the observation that children with mUPD7 experienced an earlier pubertal onset than 11p15LOM despite the higher BMI increase between 5 and 9 years in 11p15LOM is in partial contrast with the findings of Canton et al. With the limitation of the fact that our cohort includes both 11p15LOM and mUPD7 subjects while Canton's cohort includes only 11p15LOM subjects, the lack of a correlation between weight/BMI gain and the timing of puberty in our cohort is not in line with the hypothesis of the key role of a marked BMI increase as causative of early puberty in SRS (16). Although GH administration does not appear to have a negative impact on the progression of puberty (our patients are treated with similar GH dosage) and testicular volume (36) and that of GnRH analogs on increasing BMI (37), we cannot completely rule out their role in our cohort. However, it should be considered that GH treatment has never been performed in 3 out of 5 SRS subjects with precocious puberty in our cohort.

The lack of a correlation between weight and BMI gain and pubertal onset in our cohort as well as the emerging data in literature on the role of imprinted genes in pubertal timing (38, 39) may suggest that imprinted genes involved in SRS can play a role in the timing of puberty. While it is known that the time of puberty has a strong genetic component, recently epigenetics has been implicated as an important regulatory mechanism underlying not only the developmental process by which GnRH release is first kept in check before puberty, but also the increase in GnRH secretion (38). Loss of function mutations in Makorin Ring Finger Protein 3 (MKRN3), a maternally imprinted gene on chromosome 15, are identified genetic causes of central precocious puberty (39). More recently,

several mutations in a second maternally imprinted gene, Delta-like noncanonical Notch ligand (DLK1) have also been associated with central precocious puberty (39). Given the role of mutations in the imprinted genes MKRN3 and DLK1 in pubertal timing, other imprinted candidate genes should be considered for a role in puberty initiation (39).

The two SRS boys with biochemical parameters of precocious puberty had small testes. This characteristics has been previously described in a study including 11 SRS boys (6 11p15 LOM and 5 idiopathic SRS) and can be the sign of an impaired gonadal function (40). Taking this finding into account, the assessment of testicular size in these children could underestimate the pubertal stage making it unreliable for the estimation of pubertal development in this group of patients. Low levels of inhibin B, indicating Sertoli cell dysfunction, have been reported by Goedegebuure et al. in 4 out of 14 post-pubertal SRS (26). Our study has strengths and limitations. The number of patients for a rare disease and the strict diagnostic criteria (clinical diagnosis according to NH scoring system confirmed by molecular analysis) represent the strengths, while the small number of sex-related molecular subtypes and the different mean age of SRS and SGA groups are limitations.

The different mean age of SRS and SGA groups can be explained by the strict diagnostic criteria as well as by the fact that while the SRS subjects are referred to our University Center since their first months/years of life, SGA subjects are often followed at the local hospitals and consequently they often come to our attention late. In conclusion, SRS children should be carefully monitored for signs of early puberty onset. It should be considered that SRS boys may have testicular hypoplasia and sex hormone assessment could be valuable. Since testicular hypoplasia can be a sign of gonadal disfunction, measurement of inhibin B and anti-mullerian hormone could be useful in SRS male adults. Understanding the pubertal characteristics in SRS can help define the best preventive measures (avoiding overfeeding by nasogastric tube, close pubertal monitoring and bone age assessments) and the appropriate time window for therapeutic intervention (aromatase inhibitors, GnRH analogs) when needed. Additional data are required to better understand the potential role of epigenetics in puberty and gonadal function as well as the impact of BMI gain on pubertal onset, adult height and long-term metabolic outcomes.

## Data availability statement

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

## Author contributions

GP designed the study, examined the clinical phenotypes of the patients, took care of patients' follow-up, collected data, drafted and revised the manuscript. FM took care of patients, collected data, drafted and revised the manuscript. MC performed the statistical analyses. MSca, MSch, EC helped in following the patients and in data collection. SR performed molecular analysis. DF, MB, FN, AA, GD'A, RG helped in following the patients. MM designed the study, drafted and revised the manuscript. NDI designed the study, drafted and revised 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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# Corrigendum: Pubertal timing in children with Silver Russell syndrome compared to those born small for gestational age

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KEYWORDS

**puberty, bone age, silver russell syndrome, 11p15 LOM, mUPD7**

A Corrigendum on

Pubertal timing in children with Silver Russell syndrome compared to those born small for gestational age

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**Incorrect Affiliation**

In the published article, there was an error in affiliation 5. Instead of "Cytogenetic and Molecular Genetics Laboratory, Istituto Auxologico Italiano, Milano, Italy", it should be "Cytogenetic and Molecular Genetics Laboratory, IRCCS, Istituto Auxologico Italiano, Milano, Italy".

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

**Incorrect Funding**

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# MKRN3 role in regulating pubertal onset: the state of art of functional studies

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Puberty is a critical process characterized by several physical and psychological changes that culminate in the achievement of sexual maturation and fertility. The onset of puberty depends on several incompletely understood mechanisms that certainly involve gonadotropin-releasing hormone (GnRH) and its effects on the pituitary gland. The role of makorin ring finger protein 3 (MKRN3) in the regulation of pubertal timing was revealed when loss-of-function mutations were identified in patients with central precocious puberty (CPP), which to date, represent the most commonly known genetic cause of this condition. The *MKRN3* gene showed ubiquitous expression in tissues from a broad spectrum of species, suggesting an important cellular role. Its involvement in the initiation of puberty and endocrine functions has just begun to be studied. This review discusses some of the recent approaches developed to predict MKRN3 functions and its involvement in pubertal development.

KEYWORDS

**MKRN3, central precocious puberty, functional studies, E3 ubiquitin ligase, epigenetic, auto-ubiquitination**

## Introduction

Puberty is a complex developmental process through which organisms acquire sexual maturity, characterized by the acquisition of secondary sexual characteristics, the maturation of the gonads, and the attainment of reproductive capacity (1). From a purely neuroendocrine perspective, puberty begins with an increased pulsatile release of GnRH from neurosecretory neurons, scattered from the pre-optic area (POA) to the

caudal hypothalamus, which induces increased production of LH and FSH by the pituitary gland necessary for gonadal function. In humans, GnRH has a pulsatile secretion during the fetal life and mini-puberty, followed by a period of quiescence during childhood and a reactivation of its secretion at puberty (2). Systems biology approaches have recently suggested that the control of puberty and its timing is regulated by multiple sets of genes/proteins and requires the involvement of several mechanisms that can coordinate the hierarchical activation/deactivation of stimulatory and inhibitory neuronal pathways (3). A considerable number of studies over the past decades have investigated these neural networks, and several neuropeptides and neurotransmitters have been identified in the intricate balance between inhibitory and excitatory inputs to GnRH neurons. Among these, kisspeptin (encoded by the *KISS1* gene), neurokinin B (encoded by the *TAC3* gene) and glutamate exert excitatory functions and are critical for pubertal activation of GnRH neurons. Loss of function mutations in some of these neurotransmitters or alteration in their signaling lead to hypogonadotropic hypogonadism, while very rare gain of function mutations cause early reactivation of GnRH secretion and central precocious puberty (CPP) (4).

CPP is characterized by the gonadotropin-dependent development of secondary sexual characteristics before the age of 8 in girls and 9 years in boys. In 2013, loss of function mutations in *MKRN3*, a maternally imprinted gene that encodes makorin RING-finger protein-3, have been identified in five families with CPP unrevealing a new inhibitory component of the gene regulatory network that governs the onset of puberty (5). In particular an increasing number of deleterious mutations has been described in both familial and apparently sporadic cases, making MKRN3 deficiency the most frequent genetic cause of central precocious puberty with a prevalence ranging from 0.5-17.5% in sporadic cases to 9-46% in familial cases (6, 7). The human *MKRN3* gene is composed of a single exon and is located on the 15q11.2-q13 chromosome, in the critical region of Prader Willi syndrome and it is subjected to maternal imprinting. Recent genome-wide association studies (GWAS) reported associations between several paternally inherited *MKRN3* variants and the age at menarche, highlighting its important role during puberty onset (8). Furthermore, a peripubertal decline in the serum levels of MKRN3 has been documented in both sexes (9–11) and different studies have shown that the hypothalamic expression of MKRN3 mRNA and protein is significantly reduced before the onset of puberty in mice, suggesting MKRN3 potential relevance of the repressive actions in the central control of puberty (5). Although the above evidence collectively supports a relevant inhibitory role of MKRN3 in pubertal onset, the molecular mechanisms and/or regulatory elements responsible for its precise control remain unclear. This review presents a state-of-the-art of the *in vivo* and *in vitro* functional studies focusing on MKRN3 potential mechanisms of action.

## MKRN3 protein structure and expression in animals and humans

*MKRN3* was the first member of the makorin gene family identified in 1999 by Jong et al. (12) as one of several maternally imprinted genes located in the critical region of Prader-Willi syndrome (PWS) of human chromosome 15q11.2-q13.

Like all other makorin proteins, MKRN3 has a distinctive organization. Specifically, MKRN3 consists of a centrally located RING finger motif (C3HC4), two amino-terminal C3H zinc finger motifs followed by a unique pattern of conserved Cys-His residues called Makorin zinc finger motif, and a carboxy-terminal C3H zinc finger motif. From this specific structure, MKRN3 functions can be predicted. The function of the unique Cys-His makorin motif is still unknown, but it has been suggested to be a DNA binding domain. The C3H zinc fingers have been found in a variety of ribonucleoproteins suggesting an RNA-binding function that can be involved in post-transcriptional RNA processing at multiple levels, including alternative splicing, mRNA stability, mRNA localization and translation efficiency. The RING finger domain is found in most E3 ubiquitin ligases and mediates protein enzymatic activity of the protein by transferring ubiquitin from an E2 ubiquitin-conjugating enzyme to target protein substrates (13). These modifications have a range of biological effects on the target protein substrate, from proteasome-dependent proteolysis to post-translational control of protein function, structure, assembly, and/or localization. In fact, E2-E3 complexes can monoubiquitinate a lysine substrate altering the cellular non-proteolytic functions by changing protein stability or localization or synthesizing a polyubiquitin chain of lysine residues driving proteolytic processes (14, 15).

MKRN3 is highly conserved between species, in fact amino acid sequences in mice and humans share approximately 69% identity and 82% similarity with the highest level in the RING and 3' UTR region implying important functional significance for these regions of the protein (12, 16). Although MKRN3 is ubiquitously expressed in adult human tissues, a previous study in mice showed that Mkrn3 is highly expressed in the hypothalamic arcuate nucleus (ARC), where a group of neurons, named KNDy (Kisspeptin/Neurokinin/Dynorphin), are located and considered critical regulators of GnRH secretion (Figure 1). The inverse correlation between *Mkrn3* expression and *Kiss1* and *Tac3* in ARC before the onset of puberty suggests that Mkrn3 may play a role in the inhibition of GnRH secretion during the quiescent prepubertal period, probably acting at the hypothalamic level to inhibit stimulatory inputs (17). Phenotypic studies have shown that

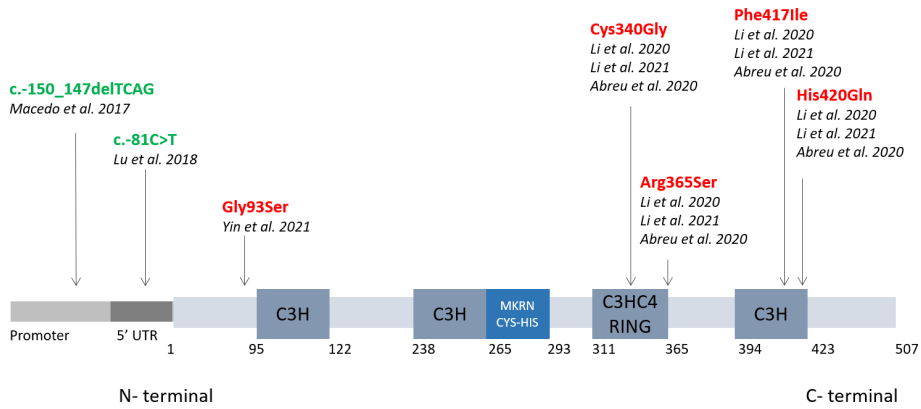


FIGURE 1
CPP-associated mutations compromise the auto-ubiquitination and binding affinity of MKRN3. The image shows the structure of the MKRN3 gene, which consists of three zinc finger domains (C3H) and one RING finger domain (C3HC4). The major mutations tested in functional studies are shown here with a black arrow indicating their location on the MKRN3 protein. Mutations causing defects in MKRN3 expression are shown in green, while in red are shown mutations affecting auto-ubiquitination or binding of MKRN3 to promoters of effector genes.

*Mkrn3* knockout mice present many symptomatic features of human CPP while also showing 50% higher expression of GnRH1 mRNA than wild-type controls. In GT1-7 cells, derived from GnRH-positive hypothalamic neurons, mouse MKRN3 potently repressed GnRH1 expression at both mRNA and protein levels (18). This inhibition may be due to ubiquitin-dependent protein degradation mechanisms or the direct involvement of Mkrn3 in transcriptional repression, as already demonstrated for Mkrn1, which appears to inhibit RNA polymerase II-dependent activators through a transcriptional interference or "squelching" process (19).

Furthermore, data reported by Human Protein Atlas indicate that MKRN3 is expressed mainly in the plasma membrane and in the cytoplasm, but also in the nucleus. Based on its location in the plasma membrane, it can be speculated that MKRN3 may also be involved in endocytosis and receptor down regulation, as has been shown for some other E3 ligases (20).

# Function through mutations effect

## Phenotype of patients with MKRN3 mutations

The MKRN3 mutations are the most common form of familial CPP. A recent study by Seraphim et al. summarized the phenotype of girls and boys with MKRN3 defects (21).

The clinical picture is indistinguishable from idiopathic CPP. The median age at the time of onset in girls is 6.2 ± 1.2 years while in boys it is 7.1 ± 1.5 years, confirming data from a recent meta-analysis and the study by Bessa focusing on male CPP (6, 22). Patients with CPP caused by MKRN3 defect had higher levels of FSH compared to those with idiopathic CPP.

Regarding the genotype/phenotype correlation, severe mutations (stop codon or frame shift mutations) were associated with greater bone age advancement than missense variants (21).

Finally, studies that reported data on adult patients with MKRN3 variants treated with GnRH analogs showed a good response to treatment (21, 23).

## Ubiquitination and autoubiquitination activity

Ubiquitin modification of many cellular proteins targets them for proteasomal degradation. A notable feature of RING E3 ubiquitin ligases is that the enzymatic activity of the E3 ligase can be monitored through ubiquitination of the protein *in vitro*. In fact, as well as promoting the addition of ubiquitin residues on target proteins, E3 ligases regulate their own stability within the cell through auto-ubiquitination, the process by which E3 enzymes catalyze the addition of polyubiquitin to themselves. In this context, MKRN3 could be considered a specific target because it contains a RING finger motif that has an active site for an E3 ubiquitin ligase involved in both autoubiquitination and substrate ubiquitination reactions (15, 24).

To this end, Li et al. (18) assembled an *in vitro* ubiquitination system to contain ATP, an E1 Ub-activating enzyme, MKRN3 as E3 ligase and different E2 Ub conjugating enzymes to support MKRN3 auto-ubiquitination. Affinity purification assays indicated that the recombinant MKRN3 protein interacted directly with E2 enzymes supporting MKRN3 autoubiquitination. Diagnostic

bands detected for MKRN3 by immunoblots or staining with Coomassie Blue on gel showed a profile of MKRN3 compatible with ubiquitinated proteins, similar to a smear. Thus, it was clear that wild-type *MKRN3*, like many other RING-type E3 ligases, could undergo autoubiquitination. When similar *in vitro* ubiquitination reactions were performed with wild-type MKRN3 or mutants (Cys340Arg, Arg365Ser, Phe417Ile, His420Gln) (Figure 2), it was evident that disease-causing mutations in the RING domain or C-terminal region compromised autoubiquitination of MKRN3 (18). These data suggested that CPP-associated MKRN3 mutations may stabilize the MKRN3 protein by altering the activity of E3 ligase (17, 18, 25). This would lead to a subsequent reduction in autoubiquitination and a slower proteasome-mediated degradation. In addition, MKRN3 mutants showed weaker suppression of GnRH1 promoter transcriptional activity than MKRN3 wild-type (18). The same MKRN3 mutations associated with CPP were tested by Abreu et al. who showed similar expression levels both for wild-type or mutants into HEK293T cells after plasmids transfection of wild-type and mutants MKRN3 and subsequent western blot analysis. Interestingly, a dramatic decrease in ubiquitination was observed in MKRN3 mutations localized in the RING finger (Cys340Arg, Arg365Ser) compared to a weaker decrease for mutants localized in the zinc finger (Phe417Ile, His420Gln), even with similar levels of immunoprecipitated protein, highlighting the importance of the integrity of the RING finger in E3 ubiquitin ligase activity (17).

Thus, autoubiquitination can lead to proteasome-dependent degradation and can be considered an essential regulatory mechanism that adds another piece to the understanding of MKRN3 homeostasis

## Defects causing reduced *MKRN3* expression

Most published studies describe causative mutations in the coding region of the *MKRN3* gene, and only a few studies recently reported defects in the regulatory region. In 2018, two groups identified in patients with CPP a small deletion (c.-150_-147delTCAG) in the promoter region of the *MKRN3* gene (26) and a single nucleotide substitution in the 5' UTR(c.-81C>T) (27) both associated with a reduced promoter activity of the gene in transfected cells (Figure 1). Accordingly to these data, regulatory region of *MKRN3* was started to be screened and new mutations have been identified confirming the causative of puberty onset. *In silico* analysis has predicted that these mutations may lead to the formation of new binding sites for transcription factors (e.g. SOX4) or their loss (PRDM14; HMX2;

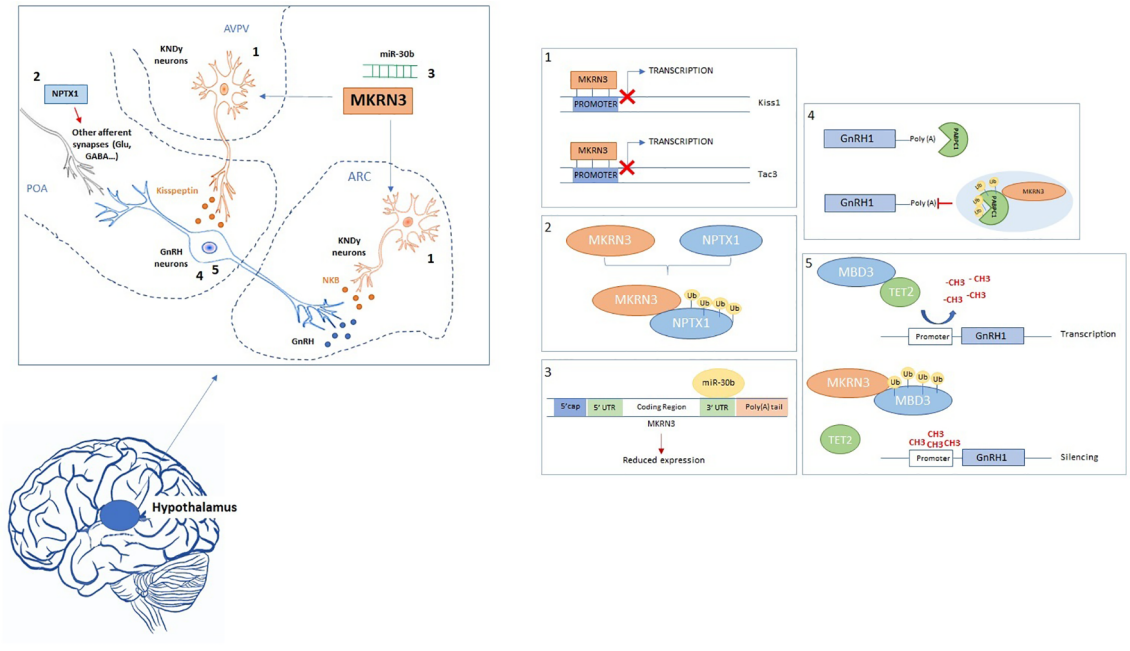


FIGURE 2
Schematic picture of MKRN3 interactions. The figure summarizes the neuronal mechanisms of action of MKRN3 and its effectors. The insert on the left shows the hypothalamus and the different nuclei that are encircled by dotted blue lines. The GnRH neuron is depicted in blue while KNDy (Kisspeptin-Neurokinin-Dynorphin) neurons are shown in orange. In the inserts on the right are represented the mechanisms of action of MKRN3 on its targets. The number of each insert report the position of the interaction on the hypothalamic neurons. Red X indicates inhibitory action. POA, preoptic area; AVPV, anteroventral periventricular nucleus; ARC, arcuate nucleus.

MTE; DREAM) with a consecutive reduction in the expression levels of MKRN3 associated phenotypically with CPP (26, 28).

## MKRN3 and its targets

The mechanism of action of MKRN3 in the onset of puberty is still unclear, but some studies have begun to report investigations into possible targets of its action.

Mass spectrometry analyses performed on a stable HEK cell line expressing MKRN3 revealed 81 novel protein interaction partners of MKRN3 that are involved in various cellular processes such as insulin signaling, RNA metabolism, and cell-cell adhesion. Among these, 20 interactors, including LIN28B, have previously been associated with age at menarche in genome-wide association studies (8). Although LIN28B appears to influence the age of menarche and infant growth, the mechanism of interaction with MKRN3 during the timing of puberty is unknown. In rats and nonhuman primates, Lin28b expression decreases in the hypothalamus at puberty, while in mice the expression of both Lin28b and Mkrn3 is reduced before puberty, speculating that it may act in concert with Mkrn3 during this stage of development. MKRN3 has also been found to interact with OTUDS, a deubiquitinase protein linked to congenital hypogonadotropic hypogonadism that can act by counteracting the effects of MKRN3 during puberty. Unfortunately, to date, there are still few functional studies on the mechanism of action of MKRN3 on its effectors.

In the following, we report the better investigated interactions.

## Direct action of Mkrn3 on expression of Kiss1, Tac3 and Gnrh1

The onset of pubertal development is a genetically controlled mechanism due to activation of the HPG axis and a subsequent increase in GnRH secretion through a complex neuronal network.

Surprisingly, while the players involved in the stimulation of GnRH neurons in puberty have been studied extensively, it is only very recently that inhibitors of GnRH, including MKRN3, have been identified.

Due to the relative inaccessibility of the human brain, the functional neuronal studies have been conducted in animal models or in engineered neurons. Using the CRISPR/Cas9 approach, Yellapragada et al. generated human-induced pluripotent stem cell (hiPSC) knockout lines for *Mkrn3* that subsequently have been differentiated into GNRH-expressing neurons. Analysis of GnRH1 expression levels showed no difference between wild-type and MKRN3-deficient cells, leading to the hypothesis that Mkrn3 is dispensable for GnRH neuron differentiation and GnRH1 expression (29). Other studies reported Mkrn3 expression may be localized into other neuronal districts involved in pubertal development and that its inhibitory action is played indirectly on GnRH secretion. Specifically, the arcuate nucleus (ARC) plays a key role in puberty control and exerts this effect through neurons that produce two key GnRH secretagogues: kisspeptin (encoded by *KISS1/Kiss1*) and neurokinin B (NKB, encoded by *TAC3/Tac3*) (30). In rodents and nonhuman primates, *in situ* hybridization showed coexpression of Kiss1 and Tac3 in ARC neurons and an increase in their mRNA levels during pubertal development has been reported (17). In parallel, the significant decrease in Mkrn3 levels in the same neurons suggests that Kiss and Tac3 may be two possible effectors of Mkrn3 inhibitory activity (17). A series of luciferase assays were performed in Neuro-2a cells transfected with vectors in which luciferase expression is driven by the human *KISS1* or *TAC3* promoter, with or without an expression vector encoding human MKRN3. Transfection of either promoter resulted in increased luciferase activity compared to the empty vector, and this activity was significantly reduced (40%-60%) by coexpression of MKRN3. MKRN3 inhibition of the activity of the *KISS1* and *TAC3* promoters did not affect the activity of the promoters of other genes involved in the stimulatory (*EAP1, Ttf1* and *Vglut2*) or inhibitory (*Viaat, Eed* and *PDYN*) control of puberty. MKRN3 then selectively represses the promoter activity of *KISS1* and *TAC3*, acting on Kiss1 neurons to reduce kisspeptin and NKB release and decrease GnRH secretion (Figure 1). Additional assays and chromatin immunoprecipitation tested the effects of some CPP-associated MKRN3 missense mutations (Cys340Arg, Arg365Ser, Phe417Ile, His420Gln) (Figure 2) revealing that only mutations located in the RING finger domain (Cys340Arg, Arg365Ser) impaired the ability of MKRN3 to inhibit the activity of *KISS1* and *TAC3* promoters but not its recruitment to these promoters.

One of the mutations located in the C-terminal domain of MKRN3 (Phe417Il) (Figure 1) showed instead a reduced binding affinity to the promoters of *KISS1* or *TAC3*, although without compromising or partially compromising its inhibitory activity. Structural analysis by X-ray crystallography and *in silico* prediction showed the intensity of the effects of different mutations in the C-terminal region of the gene, showing how a single amino acid change can alter the zinc finger conformation and its binding ability (17).

A recent mutation (Gly93Ser) reported in the N-terminal region (Fig1) showed reduced transcriptional activity of human *GNRH1, KISS1* and *TAC3* (25) suggesting an important role for the N-terminal in binding regulatory regions of genes as well. In addition, attenuated autoubiquitination activity has been reported, highlighting that this terminal region may also be involved in the ubiquitination process.

## MKRN3 and NPTX1

The Neural prentraxin-1 precursor (Nptx1) is an important protein for neuronal development and is highly expressed in the hypothalamus at the onset of puberty (31, 32) whereas a Mkrn3 decrease is observed (33). Moreover, Cummings et al. (34) reported that NPTX1 is expressed at glutamatergic synapses where it modifies glutamate release in the CNS. In that study, glutamate release increased with acute application of NPTX1. The excitatory amino acid glutamate and its N-methyl-D aspartic acid (NMDA) subtype receptor are important in the neural system that regulates sexual maturation (35). High- and multiple-dose injections of NMDA induce early puberty in rats, monkeys, and sheep. Modulation of Nptx1 levels could probably trigger this reaction thus leading to a precocious onset of the puberty. Although to date there is no direct evidence of the Nptx1 involvement in pubertal processes, the results reported by Liu et al. (33) showed a dynamic change in Nptx1 expression at different stages of the hypothalamus, indicating that Nptx1 may respond to the induction of GnRH neurons when puberty begins. Mass spectrometry and coimmunoprecipitation studies reported an interaction of Mkrn3-Nptx1 in the hypothalamus of 4-week-old mice after cerebroventricular injection of Flag-labeled Mkrn3. The RING finger domain appears to be essential for this interaction that is indeed not observed in a co-immunoprecipitation experiment using a mutant construct of Mkrn3 lacking the RING domain. The study also showed a reduction in the level of Nptx1 polyubiquitination in the hypothalamus of mice injected intracerebroventricularly with the mutant vector Mkrn3 deficient in the RING domain compared to control animals injected with the vector encoding wild-type Mkrn3. These data suggest that Mkrn3 may be able to modulate the level of Nptx1 through its E3 ubiquitin ligase activity (Figure 1) (33). The decrease in MKRN3 could therefore regulate NPTX1 activity in the hypothalamus resulting in increased glutamate release that could lead to early puberty. The interaction of these molecules appears to be a plausible hypothesis for the onset of puberty at the central level, although a recent study did not report a correlation of the level of MKRN3 and NPTX1 in peripheral blood in girls with CPP (36).

## MKRN3 and post-transcriptional modification: interaction with PABP

Recently, the critical role of MKRN3 in the regulation of post-transcriptional mechanisms involved in the initiation of puberty in mammals has emerged.

The group of Li et al. (37), identified poly(A)-binding proteins (PABPs) as potential targets of MKRN3 ubiquitination activity. PABPs are family of proteins consisting of a poly(A)-binding C-terminal domain and four RRM (RNA recognition motif) domains with different binding affinity to the poly(A) tail present at the 3' end of mRNAs. This interaction appears to regulate many aspects of mRNA homeostasis, such as stability or non-nonsense-mediated decay (NMD), stress response, control of translation initiation, and mRNA translocation (38–40).

By HEK-293 cells transfected with Flag-tagged MKRN3 and subsequent co-IP assays and mass spectrometry analysis, PABP family members, particularly PABPC1, PABPC3 and PABPC4 were shown to form complexes with MKRN3. Subsequently, GST pull-down assays and mapping-analysis showed how the central region (126-295aa) of MKRN3 interacts directly with the C-terminal region of PABPC1 (37).

Confirmation that PABPs are real substrates of MKRN3 and not mere binding partners comes from the ability of MKRN3 to conjugate poly-ubiquitin chains on PABPC1, PABPC3, and PABPC4, whereas CPP-associated mutations (Cys340Arg, Arg365Ser,His420Gln) that fall in the RING of MKRN3 or C-terminal neighboring regions have impaired this ability, with the exception of the mutant (P417). Further *in vivo* ubiquitination assays then identified four Lys residues (312, 512, 620, and 625) in PABPC1 as the major sites for MKRN3-mediated ubiquitination. Additionally, the poly-Ub chains that MKRN3 conjugates to PABPC1, could disrupt the binding of PABPC1- poly(A) through the creation of a steric hindrance, rather than directly ubiquitinating the poly(A) binding motifs as assumed for the K27 and K29 residues. All ubiquitination sites were highly conserved in different organisms (human, mouse and rat), suggesting that MKRN3-mediated ubiquitination could also play a role in the regulation of other PABPs of different species as well (37) (Figure 1).

Dual luciferase reporter assays then showed higher GNRH1 levels in MKRN3 -/- HEK293 or MKRN3 mutant constructs than in wild-type. In addition, the presence of PABPC1 appears to regulate GNRH1 luciferase expression (UTR) by stabilizing its mRNA, an effect that was almost completely abolished by reintroduction of wild-type MKRN3 but not by the CPP-associated mutant (C340G) (37).

RNA immunoprecipitation (RNAIP) and Poly (A) tail length assays collectively clearly showed that ubiquitination of PABPC1 or PABPC4 by MKRN3 negatively regulates the formation of translation initiation complex (TIC), attenuating their binding to poly (A) tail-containing mRNAs and leading to shortening of the poly(A) tail of GNRH1 mRNA (37). This could contribute to higher levels of GNRH1 mRNA and protein when *MKRN3* was mutated in CPP patients, ablated, or silenced in mice at the beginning of puberty.

## MKRN3 and the epigenetic regulation of pubertal timing

In addition to genetic determinants, epigenetic mechanisms have recently emerged as important regulators of puberty onset,

as suggested from experimental models. The elucidation of such mechanisms is still in its infancy, and, consequently, its pathophysiological implications in terms of perturbations of puberty (especially in humans) have yet to be fully characterized. Since the first half of the 1970s, DNA methylation has been described as a key epigenetic modification event involved in gene silencing. Briefly, DNA methylation and demethylation are catalyzed by DNA methyltransferases (DNMTs) and demethylases (human ten-eleven translocation methylcytosine dioxygenases, TETs), respectively. Such reactions occur at the carbon-5 position of cytosine residues in CpG nucleotides, leading to the formation or decomposition of 5-methylcytosine (5mC), 5-hydroxylmethylcytosine (5hmC) and other derivatives, which constitutes a fundamental epigenetic mechanism that regulates gene expression in mammalian cells (41). The DNA methylation profile results in a highly dynamic regulation of post-transcriptional modifications; increased methylation of 5-mC is associated with the gene promoter repression, while hypomethylation is associated with transcriptional activation. In mammalian genomes, most DNA methylation (about 70%) occurs on CpG island (42, 43). This epigenetic regulatory system is expressed in hypothalamus neurons involved in stimulating GnRH release, acting according to a transcriptional repression mechanism. In fact, in 2013, Lomniczi et al. identified a group of transcriptional silencing proteins, the Polycomb group (PcG), that repress genes responsible for the onset of puberty by preventing its premature onset (44). Increased methylation of PcG gene promoters and their reduced hypothalamic expression impair this repressive action, leading to activation of the *Kiss1* gene, thus demonstrating that in female rats the onset of puberty is regulated by epigenetic repression of the PcG complex (44).

Subsequently, experiments conducted in the basal hypothalamus using cultured GnRH neurons from rhesus monkeys showed a relationship between increased GnRH gene expression and decreased CpG methylation status, confirming the inhibitory role of DNA methylation in the timing of puberty (45). Evidence of changes in methylation has also been confirmed in humans, where distinct methylation states have been found to be associated with sex (46) and adolescent transition (47). Up to now, there are no data reporting defects in MKRN3 methylation in patients with diagnosis of CPP (48). Bessa et al. conducted the only methylome study in patients with CPP in 2018 (49) on peripheral blood leukocytes. The data obtained showed different methylation profiles in normal and precocious puberty with more than 80% differentially methylated CpG sites (DMS). These methylation changes affected several ZNFs genes, supporting the hypothesis of involvement of transcriptional repressors containing ZNF (zinc finger) motifs in human pubertal development, as previously demonstrated in nonhuman primates (49). Although a hypermethylation of *MKRN3* in pubertal or CPP subjects, no differences have been found between controls and CPP patients. Instead, the hypomethylated ZFP57 protein has been shown to be required for normal imprinting of genomic regions that control MKRN3 expression (49). No conclusions can be drawn from this single piece of data, which should instead urge the scientific community to further investigate the methylation status of MKRN3 in humans.

In addition to DNA methylation, other epigenetic regulatory mechanisms such as those mediated by microRNAs (miRNAs) are known to play key roles in the regulation of a wide range of cellular and body functions. A recent integrated analysis of DNA methylation and miRNA expression identified the influence of these genetic regulators on complex traits, such as age at menarche (50). Although the potential involvement of miRNAs in the control of MKRN3 activity has not been thoroughly studied, it has recently been reported that the 3' UTR of the Mkrn3 transcript is a key element in the post-transcriptional regulation of miRNA-mediated gene expression. This recent evidence suggests a novel and interesting mechanism of puberty regulation upstream of MKRN3.

## MKRN3 in PWS and defects in methylation state

The human *MKRN3* gene is associated to Prader Willi syndrome, which results from genomic imprinting errors with lack of expression of paternally imprinted genes located in the 15q11.2-q13 region, as previously mentioned. The genetic mechanism commonly responsible for this disorder is the deletion of a 5-6 Mb region of paternally imprinted chromosome 15 (found in 65-75% of affected individuals). The remaining individuals have a maternal uniparental disomy (UPD15), or sporadic defects in a genomic region that controls the imprinting process. Despite this syndrome has been extensively investigated, the functional and physiological relevance of *MKRN3* in PWS is still not fully elucidated although this gene is now clearly associated with developmental processes. Indeed, it is interesting to note that the hypogonadism typical of PWS manifests with incomplete or delayed pubertal development and that only in a small number of subjects is the lack of the paternal *MKRN3* allele associated with CPP (51, 52).

A recent pilot study conducted by Mariani et al. (53) reported MKRN3 detectable serum levels in half patients in a cohort of genetically confirmed PWS, regardless of the genetic etiology of the syndrome (del or UPD).

Even MKRN3 serum levels in PW subjects were lower than those typically reported in the literature for normal as well as CPP patients, this finding suggests residual expression of the theoretically silenced maternal allele or its reactivation by demethylation. Prior to this study, it was widely accepted that only the paternal allele of *MKRN3* was expressed, while the maternal allele was completely silenced. However, in the brains

of mice with deletion of the imprinting center, incomplete silencing of the genes included in the paternally inherited PWS critical region and a low level of expression of the maternal alleles were reported (54). In addition, gene expression studies by Rogan et al. reported loss of imprinting in the lymphoblasts of two PWS patients with a deletion and two atypical PWS patients with a maternal dysomy. Although these studies were not extended to brain expression profiles, the transcription of a subset of imprinted genes in some patients with PWS with maternal UPD suggested that relaxation of imprinting in these patients could be responsible for milder phenotypes (55).

Although further studies are needed, similar mechanisms could be hypothesized for all genes within the PWS region, including *MKRN3*. Potentially, analyses of human genome sequence and global methylation in patients with PWS, with or without CPP, could establish epigenetic alterations of MKRN3 in the pathogenesis of this disorder.

## MBD3

The expression of DNA methyltransferases (DNMT) and methyl binding proteins (MBP) mRNA in goats and rats showed different expression between the prepubertal and pubertal stages, highlighting the involvement of these proteins in the epigenetic regulation of puberty (56).

In particular, proteins from the methyl-CpG binding domain (MBD) family as MBD2 and MBD3 interact with members of the nucleosome remodeling deacetylase complex to suppress gene expression. MBD3 has been shown to the bind primarily to gene promoter and support TET2 activity, suggesting that it could dynamically and specifically influence the methylation status of gene loci (57).

Protein interaction assays identified the MBD3 methyl-CpG-binding protein as a physiological substrate for the E3 ligase activity of MKRN3, which was shown to conjugate poly-ubiquitin chains to lysines on multiple sites in MBD3 (18). One of these sites resides in the methyl-CpG binding domain of MBD3 whose MKRN3-mediated ubiquitination could disrupt the binding of MBD3 to the GNRH1 promoter containing 5hmC. The other major sites for such ubiquitination were mainly located in the C-terminal fragment of MBD3, which is directly involved in the MBD3-TET2 interaction, suggesting that this polyubiquitination of MBD3 would impair the MBD3-TET2 interaction resulting in a decrease in 5hmC content in mammalian genomes, but could also affect the binding of MBD3 to its target loci, such as the GNRH1 promoter. These two mechanisms would, in concert, ensure the suppressive effect of MKRN3 on GNRH1 expression, thus inhibiting the initiation of puberty. This repression imposed by MKRN3 on GNRH1 expression would be lost when the action of MKRN3 is impaired by loss-of-function mutations, as demonstrated by the *in vivo* ubiquitination assay performed on immortalized B lymphocytes derived from patients with CPP with MKRN3 defect (18). Furthermore, wild-type MKRN3 has been shown to fail to suppress GNRH1 transcription in cells with genetically ablated endogenous MBD3, suggesting the existence and synergy of the MKRN3-MBD3 complex in the regulation of hypothalamic initiation of puberty. Reduced MKRN3 activity and impaired MBD3-TET2 interaction could promote demethylation in the GNRH1 promoter and hypothalamic activation of GNRH1 transcription with the consequent onset of puberty (Figure 2).

These results demonstrate a new molecular mechanism through which MKRN3 contributes to regulate the epigenetic switch in the onset of mammalian puberty.

## Mirna: Mir30b

There is still a conspicuous lack of knowledge about the mechanisms of upstream MKRN3 regulation. Using miRNA-target prediction tools based on different bioinformatics methods, several sets of potential miRNA regulators for the MKRN3 gene were identified. In particular, three regions in the 3' UTR of MKRN3 were predicted to have a high affinity for members of the miR-30 family (i.e., miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e). In the rat hypothalamus, the expression of Mkrn3 and miR-30b displayed opposite profile during postnatal maturation and co-expression in Kiss neurons and Kiss1 cell lines (mHypoA-55). The possible repressive role of miR-30b was demonstrated by functional *in vitro* analyses. Luc-Pair miR luciferase assays cotransfecting pre-miR-30b with the reporter plasmid containing the 3'UTR of Mkrn3 showed a marked reduction in the luciferase signal (>65%) indicate that miR-30b targets the 3' UTR of Mkrn3 and drives a repressive signal to Mkrn3 expression *in vitro*. Furthermore, during the juvenile period, central infusion of miR-30 blockers that bind to the 3' UTR of Mkrn3 reversed the prepubertal decrease in hypothalamic Mkrn3 protein and delayed female puberty.

# Conclusions

The extremely early activation of HPG is referred to as central precocious puberty (CPP). Studies on this disorder are useful in unraveling the mechanisms that regulate the onset of puberty. The increasing number of reported loss-of-function mutations in the MKRN3 gene has made it the most frequent monogenic cause of CPP. Although the function of this gene is not completely known, MKRN3 appears to function as a brake on neuronal GnRH release, preventing activation of the HPG axis. A number of studies have begun to point to possible targets of MKRN3 action; indeed, mass spectrometry analyses performed on a stable HEK cell line revealed 81 new protein interaction partners of MKRN3 with high reliability. Among

these, 20 interactions were previously associated with age at menarche in GWAS studies. Functional studies, mainly based on luciferase assays and protein interaction analyses, have started to report results on possible mechanisms of action by which MKRN3 might mediate its effects. The first mechanism of action is related to its ubiquitin ligase activity. MKRN3 is in fact able to add ubiquitin chains both on its targets, by promoting their proteasome-mediated degradation, and on itself, by regulating its own expression levels. Essential for this function is the integrity of the RING FINGER domain in which the enzyme activity resides. MKRN3 has been shown to interact with the neural precursor prentraxin-1 (Nptx1) in the hypothalamus of 4-week-old mice, and the C3HC4 ring finger domain appears to be essential for this interaction. Furthermore, reduced Nptx1 polyubiquitination was found in the hypothalamus of mice injected intracerebroventricularly with the Mkrn3 mutant lacking the RING domain, which confirmed its ubiquitin-mediated inhibition. Similarly, MKRN3 modulates other effectors, such as PABPC1, involved in GNRH mRNA stabilization mechanisms, and MBD3, involved in GNRH promoter methylation. Again, mutations in the RING of MKRN3 report reduced inhibition of its targets, resulting in increased GNRH levels and the onset of puberty.

The second hypothesized mechanism of action sees MKRN3 as a transcriptional repressor. Studies on neuronal cell lines have indeed confirmed a direct interaction of MKRN3 with the promoters of genes involved in pubertal timing, such as *KISS1* and *TAC3*. The effects of some CPP-associated MKRN3 missense mutations revealed that only mutations located in the RING finger domain impair MKRN3's ability to inhibit the activity of the KISS and TAC3 promoters, but not its recruitment. On the other hand, mutations located in the N- and C-terminal domains of MKRN3 showed reduced binding affinity to the promoters of the aforementioned genes, without compromising its inhibitory activity. This supports the hypothesis that MKRN3 does not act directly as a transcriptional regulator of these genes, but rather indirectly, possibly as part of the transcriptional repressor network, as already demonstrated for Mkrn1.

Finally, epigenetic mechanisms have also been implicated in regulating the onset of puberty. In particular, very recently miRNAs have emerged as novel CPP-associated factors that contribute to the regulation of both kisspeptin and GnRH secretion. The latest evidence shows that miR-30b drives a repressive signal for Mkrn3 expression *in vitro* by binding to its 3' UTR.

As can be seen from the studies reported in this review, there are multiple ways through which MKRN3 exerts its action on puberty onset timing.

Studies on defects in this gene causing CPP in humans have helped to elucidate these mechanisms.

Further studies are needed to investigate new effectors of MKRN3 and to confirm the mechanisms of action postulated so far.

## Author contributions

SP wrote the manuscript and designed the figures. GC wrote the manuscript. FA, AP, and EM made a critical revision of the manuscript. AG supervised the manuscript and final approval. 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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# Genetic, epigenetic and enviromental influencing factors on the regulation of precocious and delayed puberty

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The pubertal development onset is controlled by a network of genes that regulate the gonadotropin releasing hormone (GnRH) pulsatile release and the subsequent increase of the circulating levels of pituitary gonadotropins that activate the gonadal function. Although the transition from pre-pubertal condition to puberty occurs physiologically in a delimited age-range, the inception of pubertal development can be anticipated or delayed due to genetic and epigenetic changes or environmental conditions. Most of the genetic and epigenetic alterations concern genes which encode for kisspeptin, GnRH, LH, FSH and their receptor, which represent crucial factors of the hypothalamic-pituitary-gonadal (HPG) axis. Recent data indicate a central role of the epigenome in the regulation of genes in the hypothalamus and pituitary that could mediate the flexibility of pubertal timing. Identification of epigenetically regulated genes, such as Makorin ring finger 3 (*MKRN3*) and Delta-like 1 homologue (*DLK1*), respectively responsible for the repression and the activation of pubertal development, provides additional evidence of how epigenetic variations affect pubertal timing. This review aims to investigate genetic, epigenetic, and environmental factors responsible for the regulation of precocious and delayed puberty.

KEYWORDS

**precocious puberty, delayed puberty, genetic, epigenetic, enviromental factors, endocrine disruptors**

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## Introduction

Puberty represents a significant period in the stages of growth and development that defines the transition from childhood to adulthood due to psycho-physical changes. In addition, the reproductive capacity is acquired. Physiologically, the start of the puberty is caused by the reactivation of signals already developed during fetal life. Indeed, the hypothalamic-pituitary-gonadal axis (HPG) activity ranges from birth to 4-6 months and 2 years, in males and females respectively (1). This phenomenon called "mini-puberty" is due to a decrease in the levels of placental sex hormones, and the resulting loss in negative feedback on gonadotropin releasing hormone (GnRH). After this period, there is a halt of GnRH pulse generator until puberty, which slows reproductive function. The mechanisms that trigger the reinitiating of the GnRH pulse generator and the inception of puberty are not yet clear, although several factors are involved in regulating pubertal timing (2, 3) (Figure 1).

Genetic background explains about 50-80% of the variability in pubertal onset and progression (4). Some ethnics groups, particularly African American and Hispanic, show an earlier onset of puberty due to genetic and nutritional factors (5). Prenatal conditions, such as intrauterine growth restriction (IUGR) and small for gestational age (SGA) birth, may affect pubertal development (6). Maternal breastfeeding appears to inhibit the early onset of puberty, mainly due to the positive effect on the childhood overweight (7). Nutritional conditions such as excess of energy intake, macro/micronutrient imbalance and dietary styles can determine the early activation of the HPG axis (7). Childhood obesity may impact on the early onset of pubertal development, albeit no statistical evidence exists on the difference in the age of menarche occurrence between obese and normal weight girls (8). Maternal education, social level, age of menarche occurrence, pre-pregnancy body mass index (BMI), ethnicity, age upon delivery, smoking habits, and alcohol/coffee/tea consumption during pregnancy, are reported to correlate with pubertal timing variations in the offspring (9). Environmental factors, such as substances capable of interfering with the endocrine system (phthalates, dioxins, polybrominated biphenyls, and polychlorinated biphenyls) seem to have a role in influencing pubertal timing (4, 10, 11). Finally, epigenetic mechanisms are assumed to have a central role in regulating the pubertal onset through a balance between repression and activation of gene expression (12).

The aim of this review is to focus on the new insights on genetic, epigenetic, and environmental regulations in the context of precocious and delayed puberty.

## GnRH pulse generator and the KNDy SYSTEM: the role of stimulatory and inhibitory signals

Pubertal timing is the result of the interaction among hormones, neuronal signals and environmental factors that begins in the earlier stage of development. This interaction leads to the activation of the HPG axis (2, 13). Different hypothalamic factors and excitatory and inhibitory neuronal signals modulate the GnRH pulse generator function (Figure 2). KNDy system, which includes kisspeptin/neurokinin B/dynorphine A (KNDy), represents the most important regulator of GnRH secretion. Kisspeptin encoded by the *Kiss1* gene and generated by Kiss1 neurons is the key element of the GnRH pulse generator, together with the neurokinin B and

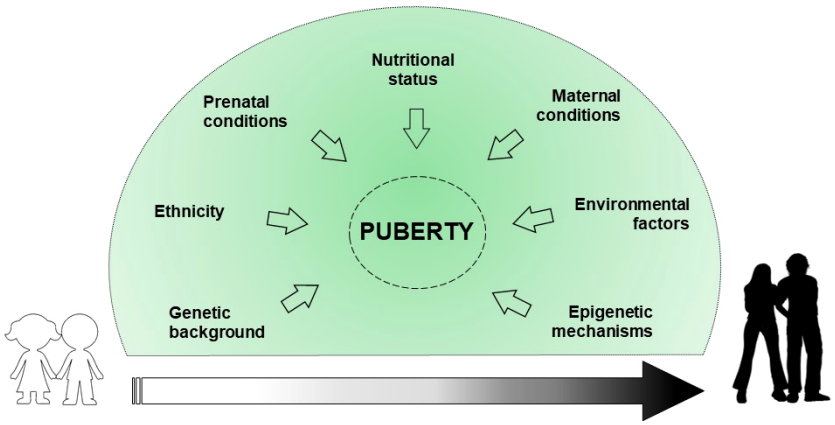


FIGURE 1
Exogenous and endogenous factors involved in the regulation of the puberty onset.

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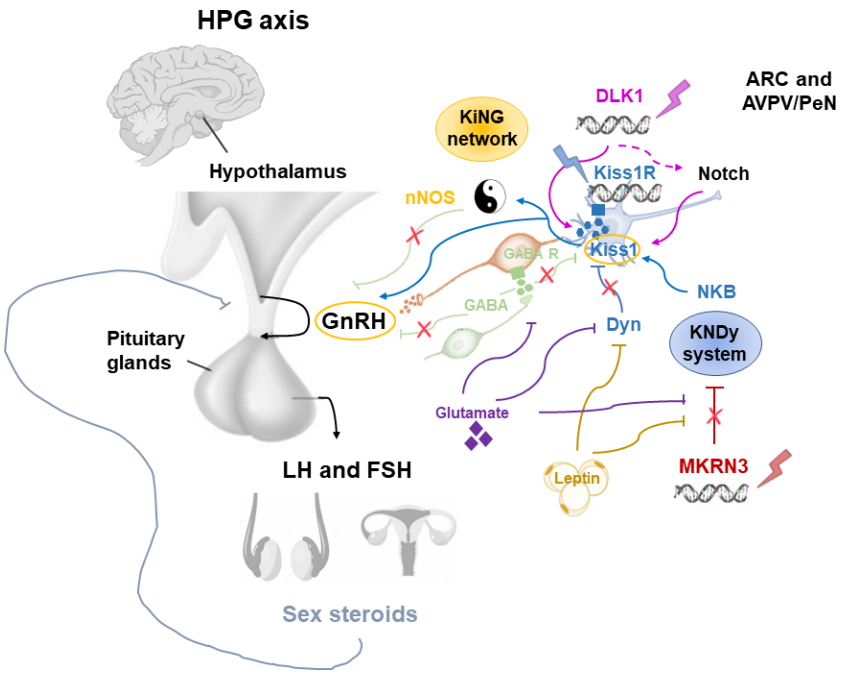


FIGURE 2
Modulation of the GnRH pulse generator by mechanisms influencing the pubertal timing. ARC, arcuate nucleus; AVPN/PeN, anteroventral periventricular/periventricular nucleus; Dyn, dynorphine A; FSH, Follicle-stimulating hormone; GnRH, Gonadotropin Releasing Hormone; HPG, hypothalamic-pituitary-gonadal; KiNG network, Kisspeptin-nNOS-GnRH; Kiss1, kisspeptin; KNDy, kisspeptin/neurokinin B/dynorphine A; LH, luteinizing hormone; MKRN3, macorin-3; NKB, neurokinin B; nNOS, neuronal nitric oxide synthase.

dynorphin A, which exert respectively stimulatory and inhibitory signals that tune kisspeptin oscillation (14). Kiss1 neurons are found in the arcuate nucleus (ARC) and in the anteroventral periventricular/periventricular nucleus (AVPV/PeN) and are controlled by sex gonadal steroids. In the females, AVPV/PeN Kiss1 neurons drive the increase in preovulatory luteinizing hormone (LH) in response to the positive feedback of estradiol. On the other hand, ARC Kiss1 neurons regulate the tonic release of GnRH/LH in response to sex steroid negative feedback, thus sending hormonal, neuroendocrine and metabolic informations (14). Kiss1 neurons has also been recognized in the posterodorsal part of the medial amygdala in mice. These neurons regulate the GnRH pulse generator, as well as influence emotional and sexual behavior, pubertal timing, and ovulation (15, 16). In humans, the role of KNDy system has been clarified by the association of loss-of-function mutations in the kisspeptin (*KISS1*), kisspeptin receptor (*KISS1R*), neurokinin B (*TAC3*), or neurokinin B receptor (*TACR3*) genes and delayed puberty and hypogonadism (17–19). On the other hand, gain of function mutations of *KISS1R* gene have been correlated with precocious puberty (20–22).

Although the KNDy system plays an essential role in the GnRH pulse generator activity, several observations showed that this is not the only system involved in the regulation of pubertal timing (23). Recently, the kisspeptin-nNOS-GnRH or "KiNG" network that is responsible for generating the "GnRH pulse" and "GnRH surge" is emerging among the regulators of pubertal development (24). In fact, nNOS and kisspeptin seem to act as the Yin and Yang, thanks to their ability to integrate and coordinate distinct signals in order to inhibit or promote GnRH secretion, respectively (Figure 2). Before the discovery of the crucial role of kisspeptin in the control of GnRH release, *in vitro* and *in vivo* studies identified the nitric oxide (NO) as a key modulator for the GnRH secretion and preovulatory GnRH/LH surge (25, 26). Neurons which express neuronal NO synthase (nNOS) are involved in the modulation of GnRH neuronal excitability and secretion. In mice, nNOS are expressed early in the hypothalamus, suggesting a role of NO in the maturation of GnRH neurons during postnatal life through the regulation of GnRH mRNA expression (27). Knock-out mouse for *NOS1* gene encoding for the nNOS resulted in hypogonadotropic hypogonadism, infertility and dose-dependent defects in olfaction, hearing, and cognition (28). Furthermore, by using a transgenic *Gpr54*-null IRES-LacZ knock-in mouse model, the expression of kisspeptin receptor GPR54 in the nNOS neurons of preoptic region of the hypothalamus has been demonstrated (29). In humans, differently to mice, some kisspeptin neurons of

the infundibular nucleus express *NOS1* (30). Recently, *NOS1* loss-of-function mutations have been found in six subjects with congenital hypogonadotropic hypogonadism (CHH), anosmia, hearing loss, and intellectual disability (30). Thus, interactions between kisspeptin and nNOS neurons may play a central role in regulating the hypothalamic–pituitary–gonadal axis *in vivo*.

Among the inhibitory signals that regulate KNDy-GnRH secretion, the Makorin Ring Finger Protein 3 (MKRN3) has a central role, as its expression in the hypothalamic ARC rapidly declines before the onset of puberty, followed by a stable decrease during the pubertal advancement (31, 32).

This is an imprinted gene as the maternal allele is silenced, and only the paternal allele is expressed. Loss-of-function mutations of *MKRN3* gene cause the most cases of familial central precocious puberty (CPP) (33–35). Furthermore, whole genome analysis studies (GWAS) demonstrated that single nucleotide polymorphisms (SNPs) of the MKRN3 region can regulate the age of menarche occurrence in healthy girls (36). Girls with *MKRN3* gene mutations show a more marked advancement in early pubertal signs and at a younger age than boys, indicating a sexually dimorphic effect of MKRN3 on pubertal development (37). Regarding the genotype-phenotype correlation, the median age at diagnosis is lower in patients with more deleterious mutations (stop or frameshift) than those with missense variants (38).

The *MKRN3* gene encodes a protein implicated in ubiquitination and cell signaling. Recently, Li et al. (39) identified the methyl-CpG binding domain (MBD) 3, an epigenetic reader which regulates gene expression, as the target of MKRN3 ubiquitination. MKRN3-mediated ubiquitination attenuates the binding of Poly(A)-binding proteins (PABPs), which regulate the stability of RNA messengers, to the poly(A) tails of mRNA. Therefore, the poly(A) tail-length of Gonadotropin-Releasing Hormone 1 (GNRH1) mRNA is shortened, and the formation of translation initiation complex (TIC) is compromised. Three members of PABPs (PABPC1, PABPC3 and PABPC4) have been identified as novel substrates for MKRN3. Thus, MKRN3 epigenetically regulates the transcription of *GNRH1* gene through conjugating poly-ubiquitin chains on MBD3 (39). The MKRN3 ubiquitination of MBD3 disrupts the interaction between MBD3 and the DNA demethylase ten eleven human translocation methylcytosine dioxygenase, 2 (TET2), as well as the MBD3 binding to *GNRH1* promoter, thus epigenetically silencing the *GNRH1* transcription and inhibiting puberty initiation (39).

Adipokines like leptin and other factors, such as glutamate and glial signaling molecules are also implicated in the control of GnRH secretion. These activator signals are enhanced by the loss of inhibitory signals within the ARC such as gamma aminobutyric acid (GABA), dynorphin A and MKRN3, resulting in positive feedback on GnRH pulse generator (23). GABA is the most important neurotransmitter which inhibits GnRH release during childhood by both indirectly acting on neurons connected to the GnRH neuronal network, or directly stimulating GnRH neurons through activation of GABA receptors alpha1-subunit (40). GABA receptors are expressed on GnRH neurons; thus, GABA antagonists increase GnRH secretion, leading to early menarche (41).

Furthermore, experimental studies have shown that the hypothalamic GABA tone inhibition leads to precocious puberty, and SNPs of the GABA signaling are related with the age at menarche (42).

## Genetic regulation of central precocious puberty

The first CPP-associated gene alteration was an activating mutation (Arg386Pro) in the G protein coupled receptor 54 (GPR54), also referred to as KISS1R, which binds the kisspeptin (20). This mutation prolongs the reactivity to kisspeptin by decreasing the degradation of KISS1R (43). Two heterozygous missense mutations in the ligand, the kisspeptin, encoded by the gene *KISS1*, were identified in unrelated subjects affected with idiopathic CPP. This variant resulted in a higher kisspeptin resistance to degradation compared with the wild type, determining greater kisspeptin bioavailability (44). On the contrary, the *MKRN3* gene, located within the Prader-Willi syndrome (PWS) region (15q11.2), is the first gene in which loss-of-function mutations have been related to CPP (34). It acts as inhibitor of the pathways leading to puberty beginning, upstream or at the level of kisspeptin and/or GnRH neurons. Low MKRN3 serum levels have been demonstrated before pubertal onset (32, 45) and in girls with CPP compared to controls (35, 46).

Like *MKRN3*, Delta-like 1 homolog (*DLK1*) is a maternally imprinted gene. It is a member of the Notch/Delta/Serrate family belonging to imprinted genes positioned on chromosome 14q32 in humans. This region is associated with Temple syndrome which is characterized by pre- and post-natal growth failure, hypotonia, motor delay and small hands. Interestingly, CPP has been described in 86% of individuals with Temple syndrome (47). *DLK1* is expressed in several tissues during embryonic development, while in postnatal life only in (neuro)endocrine tissues and stem/progenitor cells (48). The Notch signaling pathway is one of the most conserved within species, acting in a context-dependent manner by promoting embryonic cell proliferation and apoptosis (49). Notch signaling is crucial to maintaining the homeostasis in regeneration and damage repair by inducing the differentiation and transformation of mature cells (50). Several ligands and receptors are involved in Notch signaling and have specified temporal and spatial expression in

various organs and tissues, including the hypothalamus. How the Notch signaling pathway affects the onset of puberty remains unknown. Recent findings suggest that the Notch regulates progenitor cell differentiation in the pituitary gland, delaying the gonadotrope differentiation (51). The DLK1 intracellular domain has been shown to negatively regulate Notch signaling by disrupting the RBPJ-κ/Notch signaling pathway (52).

Paternally inherited *DLK1* genetic defects have been identified in four families with CPP and metabolic alterations such as obesity, early-onset glucose intolerance, type 2 diabetes mellitus and hyperlipidemia (53, 54). Moreover, *DLK1* mutation has been found to be associated with polycystic ovary syndrome and infertility suggesting a novel link between reproduction and metabolism (54).

The exact role of DLK1 in regulating the timing of puberty is not yet understood; however, DLK1 is likely to regulate hypothalamic neurogenesis and the formation of kisspeptin throughout the activation or inhibition of Notch target genes. Indeed, the Notch signaling pathway could represent a link between the *KISS1*, *MKRN3* and *DLK1* genes.

## Genetic regulation of delayed puberty

Delayed puberty (DP) consists of the absence of pubertal development from the age of 13 years for girls and 14 years for boys. The most frequent phenotype is represented by isolated and self-limiting DP (also described as constitutional retardation of growth and puberty) (55). Most of the subjects with self-limited DP have a family history of late puberty (56, 57). The self-limited DP is inherited in an autosomal dominant, autosomal recessive, or X-linked manner. Furthermore, sporadic cases are also reported. However, few patients with DP have mutations in genes causing abnormalities of the HPG axis, such as *FGFR1*, *GNRHR* and *HS6ST1*, and most of these are relatives of patients with CHH (56–58). Mutations in the Immunoglobulin Superfamily member 10 (*IGSF10)* gene have been found in six unrelated families (59). IGSF10 is important for the appropriate migration of GNRH neurons from the nose to the forebrain during embryonic development. The affected subjects showed pubertal delay without features of constitutional growth delay. A functional defect in the GnRH neuroendocrine system with an increased "threshold" for the onset of puberty, has been hypothesized.

Additionally, loss-of-function mutations in *IGSF10* gene have been found in subjects with hypothalamic amenorrhea (60), suggesting common genetic background with functional central hypogonadism. Subjects affected with both premature ovarian failure and neuronal conditions showed *IGSF10* gene mutations (61). It is not known if these patients also have deficiency of reproductive capacity or sexual lifespan.

To reinforce the concept that the alteration of GnRH neuronal migration during embryonic development can alter pubertal timing, there is a recent preclinical study which demonstrated that the deletion of neuropilin-1 (Nrp1) signaling in GnRH neurons enhances their survival and migration, and their accumulation in the accessory olfactory bulb. In female mice, these alterations result in early prepubertal weight gain, premature attraction to male odors, and precocious puberty (62).

Variants in genes associated with CHH, particularly *GNRHR*, *TAC3* and its receptor *TACR3* have been observed in in cohorts of subjects with self-limited DP (63). However, the pathogenetic role of these variants it is not known. Among other genes involved in the HPG axis function, *LEP* encoding for leptin, *LEPR* encoding for leptin receptor, and *GHSR* encoding for the ghrelin receptor could influence the pubertal timing too. Some studies identified rare variants of these genes; however, it is not clear the association with DP (56).

Pubertal timing seems to be influenced by some genes involved in energy metabolism such as *FTO NEGR1*, *TMEM18* and *SEC16B* genes that have been identified by GWAS (64). Variants of the *FTO* gene have been associated with the regulation of satiety. Rare heterozygous *FTO* variants have been discovered in pedigrees with self-limited DP combined with extreme low BMI by using next generation sequencing (65). Furthermore, knockout mice for the *FTO* gene showed significantly delayed pubertal onset (66).

During the first stages of pubertal development, the loss of the neurobiological brake is managed by several transcription factors organized hierarchically. Therefore, there are transcriptional repressions containing zinc finger motifs that can manage this complex network of genes. The best known are represented by EAP1, Oct-2, Ttf-1, Yy1. EAP1 causes the onset of female puberty through the transactivation of the GnRH promoter. One in-frame deletion (Ala221del) and one rare missense variant (Asn770His) in *EAP1* have been detected in two unrelated families. This condition would result in a reduced transcriptional activity of GnRH resulting in self-limited DP (67).

## Epigenetic control of puberty

The concept of epigenome plasticity explains the adaptation to the environment to regulate the expression of genes that can exert deep effects on the phenotype without modifying the DNA. This reactivity of the epigenome to different signals represents the "epigenetic memory". Although most of the pathways leading to such changes are still unclear, it is known that these

changes can schedule puberty to a specific stage of development (68).

There is evidence on the role of the epigenetic mechanisms in regulating the expression of key actors in the HPG axis, along with its probable role in adapting pubertal timing according to the environment (69).

## DNA methylation

The mechanisms of epigenetic regulation consist in the methylation of CpG (cytosine-guanine) DNA dinucleotides or in the modification of histone proteins (70). DNA methylation and demethylation are catalyzed by DNA methyltransferases (DNMT) and demethylases (TET), respectively, through active or passive mechanisms (71). Active demethylation is an enzymatic reaction that leads to the removal of the 5-methyl group from 5-methyl cytosine through oxidation catalyzed by members of TET family (72). TET2 promotes transcription and peptide release of GnRH thus maintaining reproductive role (73). In addition, DNA methylation and demethylation support the genomic integrity in somatic cells, across silencing or activation of transposable retroelements (REs). The role of DNA methylation in regulating the expression of KNDy system remains unclear (74, 75). DNA methyltransferase inhibitor (DNMTi) has been shown to arrest pubertal onset and this could be reversed by treatment with Kiss1 (76). There are studies showing that the onset of puberty is not regulated by changes in Kiss1 DNA methylation (77, 78). Conversely, although it is not yet clear whether *MKRN3* DNA methylation regulates pubertal onset, some studies proposed a potential role for demethylation-mediated expression of Zinc finger protein 57 (ZFP57) which regulates genomic imprinting (79). The promoter region of the ZFP57 gene is hypomethylated in pubertal girls, and its expression increases in the hypothalamus of female rhesus monkeys at the time of pubertal inception, in line with the increase in KISS1 and GNRH levels (39). Further insights into the epigenetic role of MKRN3 have recently been proven in the MKRN3 knock out mouse which displays CPP. *MKRN3* gene regulates the switch in the onset of mammalian puberty through the ubiquitination of the MBD3 which silences *GNRH1* through disrupting the MBD3 binding to the GNRH1 promoter and recruitment of TET2 (80). These observations support the role of TET2 in direct regulation of GNRH1. Another important regulator of puberty is GnRH receptor (GNRHR) which mediates the GnRH response. The *GNRHR* gene expression is regulated by DNA methylation during neuronal development (81).

Previous studies demonstrated that that Fibroblast growth factor 8 (FGF8) signaling is required for GnRH neuron ontogenesis in the olfactory placode (OP) (82, 83). FGF8 and FGFR1 deficiency is associated with Kallmann Syndrome (KS), a congenital disease characterized by hypogonadotropic hypogonadism and anosmia. Recently, it has been demonstrated that TET1, which converts 5-methylcytosine residues (5mC) to 5-hydroxymethylated cytosines (5hmC), controls transcription of *Fgf8* during GnRH neuron ontogenesis (84). This study demonstrated the importance of epigenetic-dependent timing of *Fgf8* expression during GnRH neuron emergence, and that epigenetic dysfunction can start from the ontogenesis of GnRH neurons onwards and is not limited only to postnatal GnRH neuron organization, potentially contributing to the development of CPP or DP.

## MicroRNAs

GWAS demonstrated an association between menarche age occurrence and *LIN28B* gene polymorphisms, providing the first evidence of an association between miRNAs, epigenetic regulators of gene expression, and pubertal onset (36, 85). Lin28B, and its related Lin28A, are RNA-binding proteins which inhibit the processing of miRNAs of the let-7 family. The role of Lin28 has been confirmed by functional genomic, as transgenic mice overexpressing this protein had overt DP (86). However, the exact repressive mechanism of Lin28 proteins on pubertal development is not known. Furthermore, it is not clear the eventual role of let-7 miRNAs in the central control of puberty.

It has been demonstrated that a microRNA switch regulates the increase in hypothalamic GnRH production before puberty, thus if this event does not occur accurately it may lead to the loss of GnRH expression or alteration of the rhythm of GnRH release and cause hypogonadotropic hypogonadism and infertility in mice (27). Two critical factors of this switch, miR-200 and miR-155, regulate GNRH1 expression through post-transcriptional control of ZEB1 and CEBPB expression, which in turn exert a role in GNRH1 transcriptional repressors in GnRH neurons (27). Recently, in a model of Down syndrome (Ts65Dn mice) the GnRH control appears to be related to an imbalance in a microRNA-gene network which regulate GnRH neuron maturation and hippocampal synaptic transmission (87). Considering the previously mentioned studies on *NOS1* gene alterations, in which both mice and humans show comorbidities such as sensory and cognition impairments, which can be corrected in mice at minipuberty, it can be hypothesized that the maturation of the GnRH system may also play a role in brain development in general, as well in the development of the HPG system.

In addition, miR-7a2 controls the development of the murine pituitary and the function of the HPG axis in mice; thus, its deletion leads to hypogonadotropic hypogonadism and infertility (88). Recently, the expression of miR-411-3p, miR-

382-5p, and miR127-3p has been demonstrated to contribute to variability in age at menarche (89).

## Endocrine disruptors and pubertal timing

Endocrine disrupting chemicals (EDCs) are considered responsible of changes in pubertal time (90). Several elements have been recognized as possible EDCs, such as polybrominated biphenyls, bisphenol A (BPA), atrazine (herbicides) (90–92). EDCs can interfere with reproductive functions by mimic or block endogenous hormone function, or by competing with endogenous hormones to bind to carrier proteins (93). Furthermore, they act through G protein-coupled receptors (GPRs) by altering gene expression as well as intracellular signal transduction (94, 95). A relation between early exposure to EDCs and alteration in pubertal timing or concentrations of circulating reproductive hormones has been observed (96–100). They can act in various time windows of development. During fetal life, EDCs can cross the placenta *via* passive or active transport (101–103). The exposure of zebrafish embryos to 17a-ethinylestradiol (EE2) or nonylphenol (NP) disturbs the ontogenesis of GnRH neurons in the forebrain *via* estrogen-receptor pathway (104). In rodents, GnRH neurons use a prostaglandin D2 receptor signaling mechanism during infancy to recruit newborn astrocytes which guide them into adulthood. It has been demonstrated that the exposure to bisphenol A damages postnatal hypothalamic gliogenesis and disrupts the GnRH neurons, impairing minipuberty and delaying the acquisition of reproductive capacity (105). Moreover, epigenetic alterations in testis and other systemic consequences have been observed in pregnant rodents after EDC exposure (106).

Another critical window for EDC exposure is the period of puberty. Kisspeptin neurons are particularly sensitive to early EDC exposure, as mice exposed to low doses of BPA show a reduction in these neurons and a reduced expression of KISS1 and TAC2 in ARC (107). In addition, variations in the pubertal progression have been observed in rodents exposed to EDC during puberty (108, 109).

The exposure to dibutyl phthalate in female rats affects hypothalamic kisspeptin/GPR54 expression determining early puberty and higher levels of serum estradiol (109). Furthermore, EDCs may indirectly damage the transcriptional control of gene expression (110). In pubertal boys and girls, high levels of phthalates in urine have been related with epigenome modifications such as higher DNA methylation levels in the promoter region of the thyroid hormone receptor interactor 6 (*TRIP6*) gene, which regulates pubertal onset (111). Furthermore, children who were exposed to the estrogenic insecticide DTT and then adopted showed precocious puberty (112).

These findings would explain the transgenerational EDC effects. However, although EDCs are known to affect the organization of DNA, the mechanisms by which the epigenetic modifications induced by environmental disruptors are transmitted to the hypothalamic neurons that regulate pubertal initiation must be decoded.

## Conclusions and future perspectives

The genetic and epigenetic mechanisms underlying the physiological variation of the timing of pubertal development are complex and still only partially understood. It may be that some genes act as promoters of the pubertal process, while others act as a brake. Furthermore, early and delayed puberty share some pathogenetic mechanisms, and the epigenetic regulation of the expression of genes involved in pubertal development can begin in fetal life, or during postnatal development and in infancy, with consequent modulation of pubertal time. Reproductive function in humans adapts to adjusting environmental conditions. There are "windows of susceptibility" during the different stages of development that are particularly vulnerable to events or exposures that can determine a long-term reprogramming of the reproductive function of the adult. Furthermore, animal data demonstrate a remarkable sensitivity of the GnRH network to EDCs with the possibility of transmitting phenotypic traits across generations. Molecular and human tissue, animal and cellular models are needed to understand how epigenetic modifications lead to phenotypic variations.

Although recent findings have clarified the influence of epigenetics and mRNAs in the regulation of the pubertal onset, further efforts are needed to better understand how these mechanisms work and which is the role of metabolic and environmental influences, in particular nutritional, on the epigenome. The identification of new neuroendocrine system regulators and the development of preclinical models, together with the application of new technologies for a strict functional activation or inhibition of selected neuronal populations, will be crucial for the acquisition of a deeper mechanistic knowledge of the central systems responsible for the onset of puberty.

## Author contributions

MF did substantial contributions to the conception and design of the work and revised it critically. FU and LAM wrote the first draft of the manuscript. MC and SD wrote sections of the manuscript and prepared the figures; PG

revised critically 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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# Genetic architecture of self-limited delayed puberty and congenital hypogonadotropic hypogonadism

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Distinguishing between self limited delayed puberty (SLDP) and congenital hypogonadotropic hypogonadism (CHH) may be tricky as they share clinical and biochemical characteristics. and appear to lie within the same clinical spectrum. However, one is classically transient (SDLP) while the second is typically a lifetime condition (CHH). The natural history and long-term outcomes of these two conditions differ significantly and thus command distinctive approaches and management. Because the first presentation of SDLP and CHH is very similar (delayed puberty with low LH and FSH and low sex hormones), the scientific community is scrambling to identify diagnostic tests that can allow a correct differential diagnosis among these two conditions, without having to rely on the presence or absence of phenotypic red flags for CHH that clinicians anyway seem to find hard to process. Despite the heterogeneity of genetic defects so far reported in DP, genetic analysis through next-generation sequencing technology (NGS) had the potential to contribute to the differential diagnostic process between SLDP and CHH. In this review we will provide an up-to-date overview of the genetic architecture of these two conditions and debate the benefits and the bias of performing genetic analysis seeking to effectively differentiate between these two conditions.

KEYWORDS

**delayed puberty, congenital hypogonadotropic hypogonadism, self-limited delayed puberty, genetic test, GnRH deficiency**

# Introduction

## Definition and etiology

Delayed puberty (DP) is one of the most common clinical conditions evaluated by the pediatric endocrinologist, affecting over 2% of adolescents, and implies the lack of the first signs of pubertal development beyond the average expected age for the normal population or, when puberty has previously begun, the failure of appropriate progression. Due to the earlier onset of pubertal development in girls, puberty is generally defined to be delayed if there is absence of first pubertal signs at 13 years of age in females (breast budding or thelarche) and or 14 in males (testicular enlargement over 3-4 mL of volume), respectively. These limits of normality correspond to the mean age +2-2.5 SD of the healthy general population in Western Europe. This implies that 2.5-3% of the normal subjects will fall in the extreme tail of the normal gaussian curve, and will be classified as having DP although, as better explained below, they will likely fall in the self-limited ("benign") form of this condition. Several underlying etiologies cause pubertal delay (Table 1), including chronic disease, energy-deficit and hypo- and (in girls) hyper- gonadotropic hypogonadism (1). To date, self-limited, or constitutional, DP (SLDP) is the highly common cause of pubertal delay in early adolescence, involving around 2/3 of male and 1/3 of female with DP; a para-physiological condition where individuals start puberty late, but eventually progressing to achieve full sexual maturation. By contrast, hypogonadism becomes the more common cause of DP by the late teenage years. Hypergonadotropic hypogonadism is due to intrinsic or primary gonadal failure and can easily be discerned from other forms of DP through unstimulated reproductive hormone levels, indicated by low sex steroids and high gonadotropins. It particularly concerns females (21% of DP cases), with Turner syndrome seen in 27% of girls with premature ovarian insufficiency (POI) (2, 3) but is a very uncommon cause of pubertal failure in males (vanishing testes syndrome). Conversely differential diagnosis between SLDP and hypogonadotropic hypogonadism (HH) is often challenging, as both conditions can exhibit overlapping clinical and hormonal features (2), albeit that more careful clinical ascertainment of CHH red flags could potentially identify around 50% of the CHH cases with a high degree of probability (4).

## Clinical features

Adolescents with CHH and SLDP present similar clinical traits and hormonal status at presentation but respond very differently to "expectative management" in terms of physical development and long-term psychosexual outcomes (5, 6). DP in females is established when breast budding development is absent by the age of 13 years, while in males when the testicular volume fails to surpass the threshold of 4 mL volume (evaluated by Prader orchidometer) by the age of 14 years (7). Family history of CHH versus SLDP can be helpful, but both conditions may occur within the same family (8, 9). Regarding phenotypic red flags for CHH, these comprise reproductive and non-reproductive defects, with the former only observed in males. A history of cryptorchidism,

TABLE 1 List of conditions associated with CHH or SLDP.

| | PARA-PHYSIOLOGICAL CONDITION | DISEASE CONDITION | |
|---|---|---|---|
| CLASSIFICATION | SLDP | HYPOGONADOTROPIC HYPOGONADISM | |
| | | ORGANIC | FUNCTIONAL |
| **COMMON CAUSES** | Common familial component (associated genes: IGSF10, H6ST1, EAP1, LGR4, FTO) | - CHH (Kallmann or CHH, up to 60 genes with variable inheritance and expressivity<br>- CHARGE<br>- MPHD<br>- Acquired lesions (e.g., infiltrative lesions, tumors)<br>- Metabolic diseases (e.g., iron overload)<br>- Autommune diseases (e.g., hypophisitis)<br>- Other genetic syndromes (e.g., Prader-Willi)<br>- Iatrogenic causes (e.g., surgery, radiotherapy) | - Chronic illness<br>- Energy deficit (malnutrition, malabsorption, eating disorder, excessive exercise)<br>- Stress<br>- Drugs (opiates, cannabinoids, dopamine-antagonists and, in males, glucocorticoids)<br>- Other endocrine diseases (e.g., hyper-PRL or male Cushing's) |
| | | *n.b.* Hypothalamic amenorrhoea comprosed both energy deficit and genetic elements (Delaney A et al, J Clin Endocrinol Metab. 2021 Mar 8;106(3):e1441-e1452.) | |
| **FREQUENCY** | 1:50 | 1:5000-20,000 | |

particularly the bilateral form, together with a possible neonatal underdeveloped penis (2.5 SD below average size for healthy subjects) and/or hypospadias in males is strongly suggests pre- and post-natal gonadotropin deficiency characteristic of CHH (absent minipuberty). Indeed, a definitive diagnosis can be made through basal biochemical evaluation of the HPG axis during the postnatal weeks corresponding to normal minipuberty (10, 11). However, there are no external signs postnatally in CHH female CHHs to suggest a congenital GnRH secretion defect. However, non-reproductive CHH red flags occur in both sexes, comprising clefting of the palate and/or lips, olfactory defects, alteration of digital bones, hearing loss, colorblindness, nystagmus and bimanual synkinesis, renal and/or dental agenesis or dysgenesis (10, 11). Anosmia or hyposmia are reported in around 50% of patients with CHH, thus olfactory defects (evaluated with T2-weighted coronal MRI of the olfactory bulbs and sulci and/or a quantitative olfactory test) should be evaluated and, whenever present, considered as a reliable clue towards CHH diagnosis (12). Slow growth rate and low weight tend to suggest functional HH or SLDP (7), especially when syndromic manifestations or red flags are absent. In contrast, children with CHH exhibit regular linear growth, although delayed bone maturation, osteopenia, and osteoporosis may be observed when CHH is diagnosed later in life (13, 14).

## Hormone and stimulation test for differential diagnosis

Unlike the postnatal period, gonadotropin and sex hormone measurements cannot discriminate between SLDP and CHH in early adolescence, because gonadotropin concentrations are frequently borderline also in healthy subjects of this age (1). Frequent overnight blood sampling and LH pulse-analysis has been considered for the differential diagnosis, although similar pulsatile "fluctuation" was observed in patients with SLDP, CHH and in normal prepubertal children (15). Moreover, this technique can't realistically be into routine clinical practice. In addition, the diagnostic efficacy of a single basal gonadotropin concentration it is inadequate (16, 17). A similarly low diagnostic power is offered by dynamic gonadotropin testing with GnRH or GnRH analogs (18). In fact, the lack of a gonadotropin reaction to GnRH stimulation can only confirm the absence of puberty onset, but it is not sufficient to provide a true differential diagnosis between SLDP and CHH (19). Measurement of Inhibin B, Anti-Mullerin Hormone, AMH, INSL3, and testosterone after hCG stimulation have been proposed to guide differential diagnosis (20). Although the data are promising (18, 21), they are not sufficient to support the clinical value of these newer endocrine markers. The use of new ultrasensitive methods (LC/MS) for determining hormone levels might, however, unravel new diagnostic perspectives. The testosterone response to long-term hCG stimulation and peak serum FSH response to GnRH were found to be significantly different in CHH patients (22), but there are potential long-term drawbacks to long-term hCG therapy in males who are FSH-naïve, in terms of promoting differentiation of a limited pool of Sertoli and germ cells before they have a chance to proliferate under FSH stimulation. Moreover, these methods are too inconvenient and expensive to be useful first-line approaches. Nevertheless, hormonal responsiveness to kisspeptin in boys with delayed puberty appears to be a promising new hormonal marker, although currently further studies are needed (23).

## Low dose sex steroid "priming" test

Recent studies have examined the diagnostic value of testosterone priming to differentiate between SLDP from CHH. Short-term testosterone therapy (oral, injections or transdermal) in boys with SLDP would prompt HPG activation, with the result of enlargement of the testis and rise in the production of endogenous testosterone (24). This proposed "diagnostic test" would speed up diagnosis and consequent treatment of SLDP patients, with benefits on growth velocity and virilization (25). Subjects not responding to testosterone priming (i.e., CHH patients) (24) could be than efficiently analyzed with the more expensive tests described above or offered more sustained testosterone treatment. Estradiol priming has also been proposed for HPG activation in SLDP females (2). Although, as discussed in previous paragraphs, clinicians could benefit of multiple clinical and biochemical tests to produce the diagnosis, none of these could accurately discriminate between those patients who will naturally pass and progress normally during puberty (i.e. SLDP) and those who will likely require lifelong medical therapy (i.e. HH) (2, 3). The clinical distinction between SLDP and CHH in early adolescence is of crucial importance as if CHH is diagnosed, prompt drug treatment is mandatory for puberty induction (26–28), with combined gonadotropin therapy having the potential to optimize fertility potential and quality of life in males (29).

# Genetics of CHH

CHH is defined by the diagnosis of gonadotropic deficiency throughout the infant mini- puberty or when puberty is absent or arrested in adolescence (30), although the median age at diagnosis and effective treatment of CHH remains unacceptably high around 19 years (5) and many patients present much later in adult life with sexual dysfunction, infertility, anaemia, myopathy or osteoporosis (31). CHH accounts for 24 to 85% of stable hypogonadotropic hypogonadism and includes normosmic subjects (nCHH) and subjects with olfactory defects identifying the Kallmann Syndrome (KS). KS results

from mutations in genes acting in the development of olfactory neurons; nCHH can also be underpinned by the same "neurodevelopmental" genes but is more commonly associated with mutations of genes that regulate GnRH secretion. Currently, the utility of this dichotomous division in targeting genetic testing is reduced by the clinical and genetic overlap between the two conditions (32). In CHH, a genetic basis can be identified in around 50% of patients (14, 33, 34), although as more genes are identified it has become apparent in many patients that the genetic variant originally believed to fully explain their condition did not in fact represent the whole story. So far, mutations in more than 60 genes have been classified as genetic cause of CHH, whether nCHH, KS, or both (Table 2), with few rare loci also involved in complex syndromes such as CHARGE (35–38).These genes include *ANOS1*, FGF receptor 1 (*FGFR1*), FGF8, prokineticin 2 (*PROK2*), prokineticin 2 receptor (*PROKR2*), CHD7, NMDA receptor synaptonuclear signaling and neuronal migration factor (*NSMF*), *GnRH1*, GnRH receptor (*GnRHR*), *KISS1*, *KISS1R*, tacykinin 3 (*TAC3*), *TACR3*, semaphorin 3A (*SEMA3A*), SRY-box 10 (*SOX10*), IL-17 receptor D (*IL17RD*), FEZ family zinc finger 1 (*FEZF1*), WD repeat domain 11 (WDR11), heparin sulfate 6-O-sulfotransferase 1 (*HS6ST1*), and *FGF17*. These are key genes for regulating GnRH neuronal migration and differentiation, GnRH secretion, or its upstream or downstream pathways (Figure 1). GnRH neuroendocrine cells originate in the olfactory placode outside the central nervous system and subsequently migrate into the brain during

TABLE 2 List of genes implicated in CHH.

| Gene symbol | OMIM | Inherit-ance | Olfactory defect | Main phisiological mechanism | Functionally validated variants |
|---|---|---|---|---|---|
| LEP | 164160 | AR | nCHH | Mimics energy-deficit HH in the face or early-onset morbid obesity | X |
| LEPR | 601007 | AR | nCHH | | X |
| GnRH1 | 152760 | AR, olig | nCHH | GnRH function | X |
| GnRHR | 138850 | AR, olig | nCHH | | X |
| KISS1 | 603286 | AR | nCHH | GnRH neuron activatio | X |
| KISS1R | 604161 | AR | nCHH | | X |
| TAC3 | 162330 | AR | nCHH | | X |
| TACR3 | 162332 | AR, olig | nCHH | | X |
| ANOS1 | 300836 | XLR | KS | GnRH migration | X |
| HS6ST1 | 604846 | Olig | KS or CHH | | X |
| PROK2 | 607002 | AR,AD,olig | KS or nCHH | | X |
| PROKR2 | 607123 | AR,AD,olig | KS or nCHH | | X |
| SEMA3A | 603961 | AD,olig | KS or nCHH | | X |
| PLXNA1 | 601055 | AR, olig | KS or nCHH | | |
| SEMA7A | 607961 | olig | KS or nCHH | | |
| SEMA3E | 608166 | olig | KS or nCHH | | X |
| NSMF | 608137 | AR,olig | KS or nCHH | | X |
| CCDC141 | 616031 | AR, olig | nCHH | | |
| FEZF1 | 613301 | AR | KS | | X |
| DCC | 120470 | AD, olig | KS or nCHH | | X |
| ntn1 | 601614 | AD, olig | KS or nCHH | | X |
| AMH | 600957 | AD | KS or nCHH | | X |
| AMHR2 | 600956 | AD | KS or nCHH | | X |
| NDNF | 616506 | AD | KS | | X |
| SOX10 | 602229 | AD | KS | | X |

*(Continued)*

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TABLE 2 Continued

| Gene symbol | OMIM | Inherit-ance | Olfactory defect | Main phisiological mechanism | Functionally validated variants |
|---|---|---|---|---|---|
| TUBB3 | 602661 | AD | KS | | X |
| GLCE | 612134 | – | KS or nCHH | | |
| FGFR1 | 136350 | AD | KS or nCHH | GnRH neuron fate specification | X |
| IL17RD | 606807 | Olig | KS or nCHH | | X |
| FGF17 | 603725 | Olig | KS or nCHH | | X |
| FGF8 | 600483 | Olig | KS or nCHH | | X |
| DUSp6 | 602748 | Olig | KS or nCHH | | |
| FLRT3 | 604808 | Olig | KS or nCHH | | |
| sPRY4 | 607984 | Olig | KS or nCHH | | |
| KLB | 611135 | AD | KS or nCHH | | X |
| WDR11 | 606417 | AD, olig | KS or nCHH | | X |
| IGSF10 | 617351 | AD | nCHH | | X |
| NR0B1 | 300473 | XLR | nCHH | | X |
| CHD7 | 608892 | AD, AR, olig | KS or nCHH | | X |
| SOX2 | 184429 | AR | nCHH | | X |

CHH, congenital hypogonadotropic hypogonadism; KS, Kallmann syndrome; AD, autosomal dominant; AR, autosomal recessive; olig: oligogenic. X, yes.

embryonic development (39). This route offers a developmental connection between the sense of smell and the central control of reproduction, which are both affected in Kallmann syndrome. Evidence obtained in the past years (40) suggest that GnRH neurons originating from the neural crest and ectodermal progenitors migrate in tight association with growing axons of olfactory and terminal nerves. Reached the hypothalamus, GnRH neurons finally detach from their TN guiding fibers, disperse further into the brain parenchyma, and stop the migration. At birth, GnRH neurons project to the hypothalamic median eminence and release GnRH into the hypophyseal portal vasculature (41). GnRH acts *via* the GnRH receptor, which is expressed on gonadotropic cells in the anterior pituitary gland. This action elicits the secretion of the gonadotropins, luteinizing hormone and follicle-stimulating hormone which control gonadal maturation and adult reproductive physiology *via* the hypothalamic–pituitary–gonadal (HPG) axis. As with many other diseases of genetic origin, the application of next generation sequencing technology (NGS) to the diagnosis of CHH has boosted the discovery of new candidates involved in its etiology. All known forms of inheritance have been described: autosomal dominant, recessive and with variable penetrance; X-linked recessive; oligogenicity, and transmission linked to an imprinting locus. Moreover, as already reported for Fgf8 signaling system (42, 43), also interferences in the pre-hypothalamic epigenome (throught DNMTs/TETs proteins) could have major consequences on GnRH system neurodevelopment, resulting in CHH disorder. Due to the variable expressivity and incomplete penetrance of the genetic defects, together with the actual or potential impact of oligogenicity and epigenome modifications, there is a broad spectrum of phenotypes, whether with non-reproductive defects or pure neuroendocrine phenotype, and ranging from complete CHH, with LH/GnRH apulsatility and absent pubertal development (around 2/3 of cases), to partial hypogonadism with residual LH/GnRH pulsatility (low amplitude, low frequency, or nocturnal-only pattern) resulting in arrested early puberty (around 1/3 of cases), and even reversible CHH in 5 to 20% of cases patients (44). Crucially, the genetic architecture of CHH as is presently understood does not explain the 3-5-fold excess of affected males.

## Autosomal recessive forms

Isolated CHH is transmitted as an autosomal recessive trait due to mutations in three genes: *GnRHR*, for which over 60 families have been reported (45–49), *KISS1R*, with 27 identified families (45, 50) and *TACR3*, found in 20 families (45, 51–54). In these patients, the phenotype is a common nCHH, without any non-reproductive traits of disease (36). It is worth to report that for *GnRHR* mutations, responsible for about 40-50% of hereditary cases of nCHH (48), there is a broad phenotypic variability even in the same pedigree with the same mutation (55). On the other hand, biallelic mutations in *GNRH1*, *KISS1*, and *TAC3*, ligands of the above-mentioned receptors, are a rare

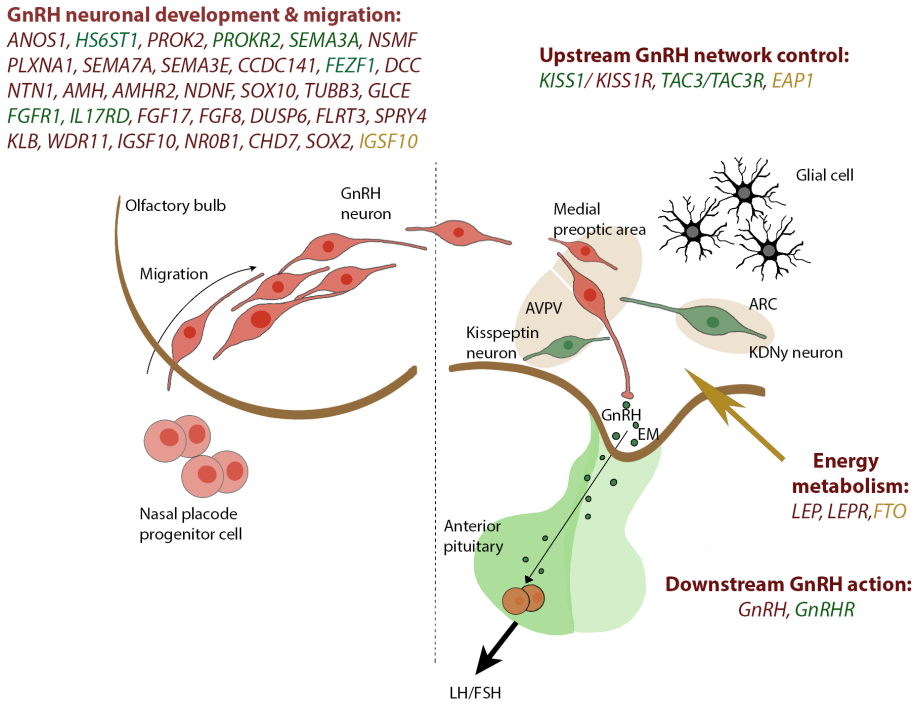


FIGURE 1
Genes reported in CHH and SLDP are related to GnRH development and GnRH function. The drawing on the left panel shows the migration of the GnRH neurons from the olfactory placode and into the hypothalamus which occurs within the first 10 to 12 weeks of rodent life. In the right panel, a concise view of our current understanding of the KISS1 neuronal networks governing GnRH secretion is depicted. Genes associated with CHH are depicted in red, shared genes between CHH and SLDP are depicted in green whereas genes related to SLDP are marked in orange. GnRH, gonadotropin-releasing hormone; KNDy, Kiss1/NKB/Dyn neuron; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus; EM, median eminence.

cause of nCHH (36). Mutations in *GNRHR* represent a paradigm of impaired functionality of GnRH, in fact, *GnRHR* encodes a receptor coupled to G proteins which determines the release of gonadotropins in the pituitary (41). *KISS1R*, *TACR3* and *TAC3* genes, encoding for a G-protein-coupled receptor for Kisspeptin, for neurokinin B and its ligand, are all instead members of a complex network where the KDNY neurons exert regulatory action on GnRH function (37).

## X-linked forms

*ANOS1* (OMIM 300836), formerly *KAL1*, is characterized by an X-linked recessive inheritance pattern; intragenic microdeletions or pathological variations of this gene have been described in 10-20% of KS (33). For canonical clinical manifestations, such as CHH and anosmia, penetrance is complete (56–61). On the contrary, other clinical manifestations such as synkinesis (56) and renal agenesis show different expressions in individuals also carrying the same variant (57–68). About 144 families have been reported so far. It is worth mentioning a female phenotype observed in 10 patients, of which one case was linked to a biallelic variant of *ANOS1* (69) and the other nine cases were traced to a mutation in another gene, indicationg a possible oligogenic inheritance. *ANOS1* encodes Anosmin1, an extracellular protein that mediates cell adhesion and play a crucial role in the migration of GnRH neurons (41).

## Autosomal dominant forms

Autosomal dominant (AD) transmission of CHH is seen more often in KS than in nCHH, with the most common genes (causing both nCHH and KS) being *FGFR1* and *CHD7*. *FGFR-1* encodes a tyrosine kinase receptor that regulates central developmental processes such as neuronal migration, fate, determination, and cell proliferation (41). *FGFR1* plays a fundamental action in the proliferation and migration of GnRH neurons to the hypothalamus as well as directly promoting olfactory bulb development. Up to now, more than 140 mutations in this gene have been described, which generally lead to a loss of function with various mechanisms (splicing, nonsense, missense, frameshift and deletions) (56, 70, 71).

*FGFR1* mutations related to KS are exemplified by incomplete penetrance (30, 72, 73) and variable clinical expression even in the same family, with patients presenting anosmia, complete phenotype, or isolated pubertal delay (72–75). Additionally, it was reported that mutations in this gene cause also nCHH (73, 76–78). Other clinical traits of *FGFR1* mutations, such as cleft lip and cleft palate, skeletal anomalies, and dental agenesis, are reported with variable frequency (30, 56, 66, 72, 79). With the discovery of other gene mutations in patients previously considered to have pure AD CHH due to FGFR1 mutations, it has become increasingly apparent that variable penetrance or oligogenicity are more common than AD inheritance with *FGFR1* mutations. *CHD7*, located in 8q12.1, encodes chromodomain helicase DNA-binding protein 7, which is expressed in different fetal tissues including the developing brain. It is one of two CHH genetic loci shared with CHARGE syndrome, a rare disorder with autosomal dominant inheritances, characterized by congenital heart disease, coloboma, choanal atresia, genital and ear anomalies, and growth and developmental retardation. Its pattern of expression reflects a potential *CHD7* contribution in the development of the olfactory bulb and GnRH neurons (41). Whereas CHARGE patients tend to harbor large *de novo* gene deletions, patients with KS and nCHH (80–82) tend to harbor missense mutations with partial loss of function that may either be *de novo* or inherited (83). Even in the case of *CHD7* mutations there is broad phenotypic variability, going from KS through nCHH to isolated anosmia (82, 83). Other clinical traits associated with *CHD7* mutations are deafness, anomalies of the outer ear and lip/cleft palate (83, 84). Indeed, it can sometimes be hard to distinguish between "mild CHARGE" and KS with multiple developmental defects. *SOX10* is a transcription factor crucial for the early development of neural crest cells, which are multipotent precursor arising from the neural tube that differentiate into different cell types. *SOX10* influence also hearing through its expression in the melanocytic intermediate cells of the cochlear stria vascularis during early development of the inner ear (85). AD *SOX10* mutations have been described in nearly 40% of KS patients with deafness (86). *PROKR2*, a gene encoding a G protein-coupled receptor and its ligand *PROK2* deserve to be reported in this section as a paradigm of mixed inheritance. The binding of *Prok2* to its receptor activates a signaling cascade with effects on the migration of GnRH neurons (41). Mutation in *PROKR2* were reported both in KS and nCHH. In 20% of cases AR inheritance pattern is reported for this gene, whereas the remaining cases are due to AD or oligogenic mechanisms (79, 87–94). *PROK2* variants, usually less frequent, can present with AD or AR inheritance patterns. As with *FGFR1*, some cases of *PROK2* and *PROKR2* mutations that were originally believed to represent AD inheritance have since turned out to be more probably oligogenic. *FGF8* encodes for a ligand of *FGF1* receptor (36). Heterozygous mutations of *FGF8* were identified both in KS and nCHH, also with oligogenic inheritance. The clinical signs include neurosensorial deafness camptodactyly and cleft lip/palate (3, 70, 95).

## Oligogenic inheritance

Recently in several cases of nCHH and KS a mutation in two or more genes, with oligogenic inheritance, has been reported. In 2006 a case of KS harboring mutations of both *PROKR2* and *KAL1* was reported (79). However, the authors did not fully appreciate the significance of their finding and, consequently, it was not until the following year that a convincing report of two different genetic variants in *FGFR1* and *NSMF* inherited within kindred causing KS only in the single individual carrying both (96). Subsequently, in 2010, Sykiotis (97) described oligogenic inheritance in 2.5% of subjects in a large series of CHH patients using a panel of just 8 genes. Successively an oligogenic mechanism in 7% (78) to 15% (32) of the subjects with CHH was documented by different groups. So far, an oligogenic mechanism of inheritance was documented at least for 16 genes (30). Reasonably, the application of NGS will increase the possibility of finding "oligogenicity" in CHH. However, the huge amount of data generated by NGS is now challenging the clear distinction between "oligogenicity" and the presence of benign variants that do not interfere with the phenotype that is determined by the variant that is principally responsible (36).

## CNV contribution to the genetic architecture of CHH

Even if more than 60 candidates have been linked with the pathogenesis of CHH, approximately 50% of cases remain genetically undetermined (37). Part of this missing genetic heritability probably resides in new candidates that require larger cohorts for their discovery or in mutations not simply detectable by whole exome sequencing experiments (WES), such as copy number variants (CNVs) and variants in the non-coding portion of the genome. CNVs are structural variants that result in either gain (duplications) or loss (deletions) of genetic material (more than 50 bp of genomic DNA). Thus far, previous investigation of CNVs with chromosomal microarrays or karyotypes in CHH has led to essential genetic findings (98–100), showing an overall prevalence of ~1% in a subset of CHH-associated genes (101, 102). However, such a low prevalence of CNVs in previous CHH studies has been imputed to the low-resolution tools deployed to call CNVs (101). To date, complex analytic pipelines now available can detect CNVs of a smaller size compared to historic microarrays, allowing precise characterization of these structural variants. In 2022, Balasubramanian and colleagues employed new validated, high-resolution CNV capture technology to examine a large

cohort of CHH patients, detecting a total CNV prevalence of 2% across 13/62 known CHH candidates (103). Although supporting the idea that CNVs in known CHH genes should be investigated in patients with CHH, this study indicates that the greater proportion of the missing heritability in CHH may relate to mutations of new coding/non-coding genes. It is hoped that, given the massive application of genome sequencing, these still elusive variants involving non-coding regions will illuminate the full genetic architecture of CHH.

## Genetics of SLDP

Pondering the distribution of puberty timing in the normal population, SLDP can be assimilated to the extreme upper limit of normality. A clear diagnosis of this condition is often difficult to achieve, even if SLDP is frequently reported in multiple generations of the same family. Most commonly, the trait is inherited in an autosomal dominant pattern, often with complete penetrance, but autosomal recessive, X-linked and bilinear pedigrees have also been reported (104, 105). Epidemiological studies of twins in both sexes have shown that the time of puberty is a highly heritable trait and that genetics play a key role in determining when healthy individuals enter puberty (106, 107). It is clear from genome wide association studies (GWAS) that many different genetic signals are crucial in the discrepancy of pubertal timing observed in the normal population (108). In contrast, one or a small number of genetic variations in each family are generally described in delayed familial puberty, with a corresponding pattern of autosomal dominant inheritance. The recent application of NGS technology to self-limited DP have unraveled fascinating new mechanisms in the genetic control of puberty (Figure 1).

### HS6ST1

Targeted and whole exome sequencing methods identified a mutation in *HS6ST1* in a broad pedigree among a large cohort of patients with isolated familial delayed puberty, without associated CHH in their relatives (84). All the family members who carried the mutation exhibited a canonical SLDP phenotype, rather than CHH; the proband entering puberty spontaneously at 14.3 years. In parallel, a mouse heterozygous knockout model found the loss of an Hs6st1 allele to cause pubertal delay in females without impairment of adult reproductive capacity. Hs6st1 +/− mice showed no impairment of fertility, development of GnRH neurons, testes, or spermatogenesis. However, a substantial delay in the timing of vaginal opening (used to determine the onset of puberty in female rodents) was observed in females. Remarkably, the Hs6st1 +/- mice had normal olfactory bulbs without any reduction in the global number of GnRH neurons in the hypothalamus or projecting to the median eminence. Consequently, the pubertal delay observed in mice could be due to variation in GnRH neuron activity or other crucial downstream pathways, controlling the expression of *Hs6st1* in both the arcuate and paraventricular nucleus (109, 110). In recent work involving 338 patients with GnRH deficiency, including 105 subjects with a positive family history, a variant in HS6ST1 gene was identified in almost 2% of patients with CHH (111). In this study, the inheritance model was complex, bypassing simple Mendelian transmission, and with substantial clinical heterogeneity suggesting a role for epigenetic mechanisms or mutations in other candidates to fully explain the observed phenotypes. In order to properly assign a role to HS6ST1 in SLDP, it is crucial to take in account the biological processes in which it takes part. Howard et al., 2018, found that reduced Hs6st1 expression and a consequent reduction of sulfotransferase activity in kisspeptin and other neurons can impact on their ability to regulate GnRH function and secretion (112). In addition, *Hs6st1* activity is a prerequisite for the correct function of *Anos1* and *Fgfr1* (111). In summary, a functionally minor heterozygous mutation might cause SLDP, whereas a more severe mutation or the simultaneous effect of a second gene (i.e., oligogenicity) could lead to a more critical phenotype such as CHH or KS.

### IGFS10

Targeted and whole exome sequencing methods identified deleterious mutations also in *IGSF10* gene. Two N-terminal variants in *IGSF10* were reported in 20 subjects with SLDP from six families, with an AD inheritance (8). Moreover, two C-terminal variants in the same gene were identified in 4 families of the same study. In one family, there was incomplete penetrance, whereas a *de novo* mutation was proposed for another family. All patients had a standard growth rate before puberty and a classic DP with a delayed pubertal spurt and normal (self-reported) sense of smell. *IGFS10* gene had hitherto never been associated with any human pathology. During embryonic development mutations in *IGFS10* impact the migration of GnRH neurons from the vomeronasal organ to the forebrain. Patients with mutations in this gene are characterized by isolated pubertal delay without retardation in growth (a pattern that is also observed in CHH). Abolition of GnRH neuronal migration due to anomalous IGSF10 signaling might determine reduced or deferred migration of GnRH neurons to the hypothalamus. This result into a functional defect in the GnRH network and a higher threshold for pubertal onset. In addition, *IGSF10* loss-of-function mutations were found in subjects with hypothalamic amenorrhea-like phenotype, suggesting a common genetic origin of functional central hypogonadism with both CHH (113) and DP. Intriguingly, mutations in *IGSF10* were recently

reported in a pedigree with a Kallmann- like phenotype and in patients with both disorders of neuronal development and premature ovarian insufficiency (114). Studies on the role of the *HS6ST1* and *IGSF10* genes in PD hypothesize that developmental defects in the GnRH system during fetal life may regulate the timing of onset of puberty in adolescence, without determining other associated phenotypic characteristics. Whether these patients will have any shortcomings in their long-term reproductive capacity or sex life span remains to be evaluated.

## FTO

*FTO* is considered the most impactful locus on BMI and the obesity risk (115). Recently, using NGS techniques, rare heterozygous variants in *FTO* gene associated with BMI and growth retardation in early childhood have been described in SLDP families (116). Moreover, mice lacking *FTO* had a significantly delayed onset of puberty (timing of vaginal opening) (116).

## EAP1

*EAP1* (*Enhanced at puberty 1)* encodes a nuclear transcription factor that trans-activates GnRH promoter, facilitating GnRH secretion, and parallelly inhibits the preproenkephalin promoter, which in turn represses GnRH secretion. Howard et al. first described human EAP1 mutations that appear to cause SLDP in 2 families (117). Affected subjects from these two families had canonical clinical and biochemical signs of SLDP, with delayed onset of Tanner stage 2 and delayed peak height velocity. Both subjects showed spontaneous pubertal development at age 18 without testosterone priming, thus excluding CHH. Two highly conserved variants, one rare missense variant in *EAP1* and one in-frame deletion, were identified in subjects with familial delayed puberty. Compared to wild-type EAP1, mutants showed a biased ability to transactivate the GnRH promoter, imputable to the diminished protein levels caused by the in-frame deletion and the altered subcellular localization triggered by the missense mutation. The same work showed that in monkey hypothalamus Eap1 binding to the GnRH1 promoter rise at the onset of puberty. These recent findings suggest that genes that determine SLDP may play a role in the redundant mechanisms that regulate the onset of puberty (e.g., number of cells migrating from the olfactory placode to the hypothalamus and pre-optic areas or modulation of GnRH function and secretion), despite genes linked to CHH that directly control the migration or function of GnRH neurons. However, further studies are needed to uncover the strongness of this intuition.

## LGR4

*LGR4* encodes a receptor for R-spondins which, once activated, potentiates the canonical Wnt signaling pathway. Through GWAS analysis *LGR4* had already been designed as a regulator of pubertal timing both in males (based on recalled age at voice breaking) and in females (based on the age of menarche (118, 119). However, mutations in *LGR4* were not previously associated with actual human disease. A recent study from Dunkel's group utilizing whole-exome sequencing of 160 individuals of 67 families in a well-characterized DP cohort identified 3 rare missense variants in *LGR4* (120). All segregated with the DP trait with an AD pattern of transmission. Specific expression of *Lgr4* at the site of GnRH neuron development has been reported. LGR4 mutants showed biased Wnt/β-catenin signaling, leading to consequences on protein expression, trafficking, and degradation. Lgr4-deficient mice showed a significantly delayed onset of puberty and lowered number of GnRH neurons compared to WT mice. In addition, we were demonstrating that *lgr4* knockdown in zebrafish embryos impact development and migration of GnRH neurons. In addition, genetic lineage tracing displayed robust Lgr4-mediated Wnt/β-catenin signaling pathway stimulation during GnRH neuron development.

# Shared genes between CHH and SLDP

It is well established the timing of puberty in normal populations has a strong genetic component (107, 119, 121, 122) even though general health, nutritional status and endocrine chemical disruptor can influence the expression of key regulators. To date, the knowledge about the genetic mechanisms that control HPG axis comes largely from reports on patients with GnRH deficiency, leading to the identification of rare variants underlying CHH. Zhu and collaborators recently addressed the hypothesis of a shared genetic basis between CHH and SLDP, performing WES analysis in 15 families with a CHH proband carrying a putative pathogenic variant in CHH genes and family members both with delayed and with normal puberty (9). A genetic origin was identified in half of relatives with delayed puberty and in in a small percentage (12%) of relatives with normal puberty. Moreover, they analyzed nearly fifty DP subjects without family history of CHH matched with controls from ExAC and identified mutations in CHH genes in 14.3% of

DP subjects and in 5.6% of controls. The heterozygous allelic variants were in *TAC3, TACR3, GnRHR, IL17RD* and *SEMA3A*. Of note, control subjects also carried potentially pathogenic variants. Moerover, Cassatella et al., 2018 determined that the genetic architecture of SLDP is closer to that of normal controls than CHH probands. Exome sequencing showed potentially pathogenic variant in CHH genes (twenty-five genes with IGSF10) in 51% of CHH patients, in 7% of SLDP probands and in 18% of healthy subjects. Oligogenic inheritance was found in 15% of CHH patients and in only 1.4% of SLDP subjects and 2% of controls. To note, potentially pathogenic variants in SLDP patients were found in *AXL, FGFR1, HST6ST1, PROKR2, FEZF1* and *TAC3* genes.

## Genetic evaluation supports differential diagnosis in patients with SLDP and CHH

As DP is a common condition in unaffected individuals, identifying a genetic cause in SLDP presents several pitfalls. Thus, a genetic variant likely to lead to this condition could have a quite high prevalence in the normal population. Furthermore, both the manifestation of pubertal delay in 10% of relatives of patients with CHH (9) and the likelihood of a spontaneous reversal in 10% of patients with CHH (123) remains consistent with a shared molecular basis of CHH and SLDP. Nevertheless, it remains true that genetic variants reported are distinct between the two diseases (32). This supports the hypothesis that NGS of a large panel of candidate genes might one day assist physicians to distinguish those adolescents with severe CHH from those with SLDP, enabling timely and correct treatment to CHH patients. Howard and collaborators studied the burden of genetic variants in an ethnically mixed cohort of adolescent patients with DP, with the purpose of validate the genetic analysis of known causative genes to confirm the diagnosis of CHH or SLDP. The results of this work show a fair correlation between patient genotype and clinical diagnosis, with a specificity of 100% and PPV of genetic tests for the diagnosis of patients with CHH. The authors also reported that subjects carrying homozygous or loss-of-function mutatons in CHH genes would probably have a final diagnosis of CHH. In contrast, patients presenting mutations reported only in SLDP with heterozygous inheritance were thought to be likely to have a final diagnosis of SLDP. These results confirm that WES testing can make a clear diagnosis of CHH for 17.4% of patients presenting with pubertal delay. This finding supports the implementation of genetic analysis in the clinical practice, in combination with clinical and biochemical observations, to validate the diagnosis of CHH in adolescents presenting with DP. It is worth mentioning that the same work identified in three patients with a clinical diagnosis of SLDP also pathogenic variants in other genes previously linked to CHH, namely *DMXL2, OTUD4* and *SEMA3*.

## Conclusions

Pubertal delay can be the presentation of a wide spectrum of clinical phenotypes ranging from CHH, which is a pathological condition and needs appropriate medical therapy, to SLDP, a possible para-physiological and benign condition mostly compatible with normal reproductive capacity post- puberty, to non-gonadal illness or energy deficit. Distinguishing between SLDP and CHH remains challenging and, thus far there is no clear and univocal evidence to assist clinicians in their differential diagnosis and management of SLDP and CHH. The availability of an accurate genetic test in clinical practice to discriminate between the two conditions has the potential for significant cost savings (preventing unnecessary investigations) and improvement of health and fertility outcomes for CHH patients.

Recently, the application of NGS technologies has shed light on the complex genetic mechanisms at the basis of CHH or SLDP, identifying new candidate genes and suggesting a different genetic backbone for these two conditions. This difference constitutes the main assumption on which to model a genetic tool that allows an early differential diagnosis between the two conditions. However, it should consider that so far only a few causal genes have been described in SLDP, leading to a lower pick-up rate for SLDP pathogenic variants in a putative diagnostic panel when compared with CHH mutations. Furthermore, variants contextualization in patients with oligogenic inheritance is difficult due to our lack of knowledge of variant–variant crosstalk. For these reasons, we suggest that so far only an integrated approach can increase the sensitivity and specificity in CHH diagnosis. Thus, we invite the readers to combine this type of genetic analysis with biochemical profiling (e.g., basal LH, FSH, inhibin B, AMH) and an accurate physical and anamnestic data collection to maximize the diagnostic accuracy. Nevertheless, in the future, as the knowledge of the genetic architecture of delayed puberty will be dramatically improved, genetic testing might offer a quick and precocious analytical tool also in clinical routine.

## Author contributions

VV, FH, and MB wrote the draft. MB, GG, SF, BC, RQ and LP revised the manuscript. VV and MB finalized 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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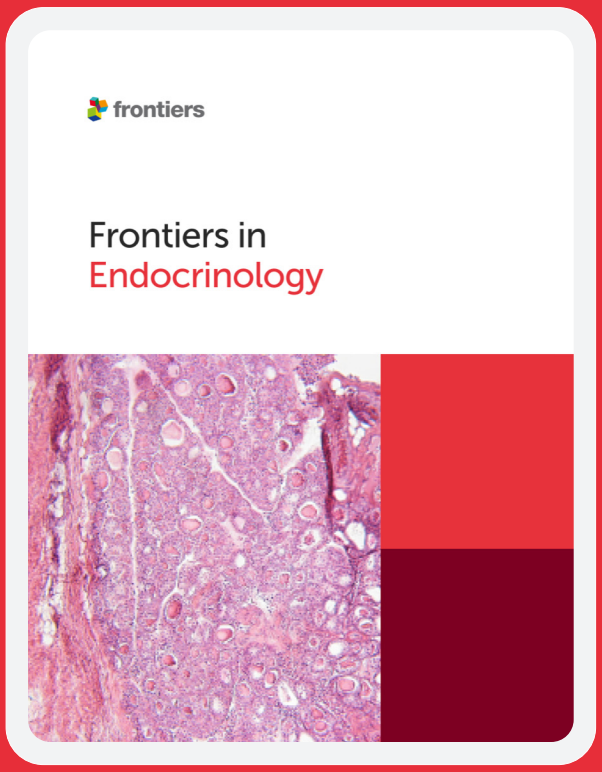


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