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Insights from the genetic characterization of central precocious puberty associated with multiple anomalies

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STUDY QUESTION: Is there an (epi)genetic basis in patients with central precocious puberty (CPP) associated with multiple anomalies that unmasks underlying mechanisms or reveals novel genetic findings related to human pubertal control?

SUMMARY ANSWER: In a group of 36 patients with CPP associated with multiple phenotypes, pathogenic or likely pathogenic (epi)genetic defects were identified in 12 (33%) patients, providing insights into the genetics of human pubertal control.

WHAT IS KNOWN ALREADY: A few studies have described patients with CPP associated with multiple anomalies, but without making inferences on causalities of CPP. Genetic-molecular studies of syndromic cases may reveal disease genes or mechanisms, as the presentation of such patients likely indicates a genetic disorder.

STUDY DESIGN, SIZE, DURATION: This translational study was based on a genetic-molecular analysis, including genome-wide high throughput methodologies, for searching structural or sequence variants implicated in CPP and DNA methylation analysis of candidate regions.

PARTICIPANTS/MATERIALS, SETTING, METHODS: A cohort of 197 patients (188 girls) with CPP without structural brain lesions was submitted to a detailed clinical evaluation, allowing the selection of 36 unrelated patients (32 girls) with CPP associated with multiple anomalies. Pathogenic allelic variants of genes known to cause monogenic CPP (*KISS1R*, *KISS1*, *MKRN3* and *DLK1*) had been excluded in the entire cohort (197 patients). All selected patients with CPP associated with multiple anomalies (n = 36) underwent methylation analysis of candidate regions and chromosomal microarray analysis. A subset (n = 9) underwent whole-exome sequencing, due to presenting familial CPP and/or severe congenital malformations and neurocognitive abnormalities.

MAIN RESULTS AND THE ROLE OF CHANCE: Among the 36 selected patients with CPP, the more prevalent associated anomalies were metabolic, growth and neurocognitive conditions. In 12 (33%) of them, rare genetic abnormalities were identified: six patients presented genetic defects in loci known to be involved with CPP (14q32.2 and 7q11.23), whereas the other six presented defects in candidate genes or regions. In detail, three patients presented hypomethylation of *DLK1/MEG3*:IG-DMR (14q32.2 disruption or Temple syndrome), resulting from epimutation (n = 1) or maternal uniparental disomy of chromosome 14 (n = 2). Seven patients presented pathogenic copy number variants: three with *de novo* 7q11.23 deletions (Williams–Beuren syndrome), three with inherited Xp22.33 deletions, and one with *de novo* 1p31.3 duplication. Exome sequencing revealed potential pathogenic variants in two patients: a sporadic female case with frameshift variants in *TNRC6B* and *AREL1* and a familial male case with a missense substitution in *UGT2B4* and a frameshift deletion in *MKKS*.

LIMITATIONS, REASONS FOR CAUTION: The selection of patients was based on a retrospective clinical characterization, lacking a longitudinal inclusion of consecutive patients. In addition, future studies are needed, showing the long-term (mainly reproductive) outcomes in the included patients, as most of them are not in adult life yet.

WIDER IMPLICATIONS OF THE FINDINGS: The results highlighted the relevance of an integrative clinical-genetic approach in the elucidation of mechanisms and factors involved in pubertal control. Chromosome 14q32.2 disruption indicated the loss of imprinting of *DLK1* as a probable mechanism of CPP. Two other chromosomal regions (7q11.23 and Xp22.33) represented new candidate loci potentially involved in this disorder of pubertal timing.

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Introduction

Human pubertal development is a complex biological process, influenced by genetic, epigenetic, metabolic-nutritional and environmental factors, in which individuals develop secondary sexual characteristics, linear growth acceleration and gonadal maturation, reaching reproductive capacity (Latronico *et al.*, 2016). Commonly, puberty starts between the ages of 8 and 13 in girls and 9 and 14 in boys. According to normal distribution, puberty is defined as precocious when pubertal development starts prior to the ages of 8 and 9 in girls and boys, respectively (Latronico *et al.*, 2016). In girls, age at menarche prior to 9 years is considered an equivalent of precocious puberty (Gomes *et al.*, 2019). The most common form of precocious puberty is the premature reemergence of the GnRH secretion with the subsequent early activation of the hypothalamic–pituitary–gonadal (HPG) axis, known as central precocious puberty (CPP) (Latronico *et al.*, 2016; Soriano-Guillén and Argente, 2019). CPP may result from congenital or acquired lesions in the central nervous system (CNS). However, most cases have no structural CNS lesions (Latronico *et al.*, 2016; Soriano-Guillén and Argente, 2019).

Lately, several studies have identified monogenic causes of CPP, elucidating genetic factors involved in pubertal timing (Teles *et al.*, 2008; Silveira *et al.*, 2010; Abreu *et al.*, 2013; Dauber *et al.*, 2017). Very rare activating mutations in the Kisspeptin (*KISS1*) and Kisspeptin receptor (*KISS1R*) genes were described in sporadic cases (Teles *et al.*, 2008; Silveira *et al.*, 2010). Inactivating mutations in the imprinted gene Makorin ring finger protein 3 (*MKRN3*) have been described as the most common cause of familial CPP with paternal inheritance (Abreu *et al.*, 2013; Canton *et al.*, 2019). More recently, loss-of-function variants in the imprinted gene Delta-like 1 homolog (*DLK1*) have also been identified as a rarer cause of CPP (Dauber *et al.*, 2017; Gomes *et al.*, 2019; Montenegro *et al.*, 2020). Interestingly, these two imprinted genes are located at chromosomal regions of (epi)genetic syndromes related with CPP to some degree. *MKRN3* is at the boundary of the region of Prader–Willi syndrome, which is associated with CPP in about 4% of cases (Cassidy *et al.*, 2012), while *DLK1* is at the locus of Temple syndrome, a rare disorder characterized by CPP in 80–90% of cases (Geoffron *et al.*, 2018).

CPP have been often described as an isolated entity in clinical practice, when other associated abnormalities may not be considered due to its complexity (Canton *et al.*, 2019; Soriano-Guillén and Argente, 2019). Despite this fact, a few studies have described patients with CPP in association with syndromic disorders (Wannes *et al.*, 2018) or other medical conditions (Winter *et al.*, 2019), but without making inferences on causalities underlying CPP. As the genetic architecture of human pubertal timing is complex, finding a genetic-molecular mechanism for each patient remains a challenge in research and clinical settings (Perry *et al.*, 2015). However, the molecular investigation of cases involving multiple systems has been a well-established approach for discovering disease genes and mechanisms, as the presentation of such patients more likely indicate a genetic disorder (de Bruin and Dauber, 2015; Wright *et al.*, 2018).

Therefore, we studied a clinically well-characterized cohort of patients with CPP associated with multiple anomalies to explore their contribution in unmasking underlying mechanisms or revealing novel genetic findings related to the reproductive disorder. The group of selected patients was submitted to a comprehensive genetic-molecular investigation, indicating possible genomic loci and candidate genes implicated in pubertal timing.

Materials and methods

Patients

In total, 197 patients (195 Brazilians, 2 Europeans) with CPP (150 sporadic cases, 47 familial cases; 188 girls, 9 boys) were submitted to a

retrospective clinical evaluation (Fig. 1). All these patients were clinically assessed by endocrinologists with expertise in genetic disorders. All of them had absence of anatomical abnormalities on magnetic resonance imaging of the CNS. The diagnosis of CPP was defined as breast or testicular volume Tanner stage 2 before the age of 8 years in girls and 9 years in boys, and basal and/or GnRH-stimulated LH levels within the pubertal range (Latronico *et al.*, 2016). Accelerated height velocity for chronological age and advanced bone age (>1 year in relation to chronological age; Greulich and Pyle method) were additional criteria. The entire cohort (197 patients) had been studied by DNA sequencing analysis (Sanger method), and pathogenic allelic variants of the coding region of genes known to cause monogenic CPP (*KISS1R*, *KISS1*, *MKRN3* and *DLK1*) were excluded. A detailed phenotypic characterization was performed in all patients including analysis of inheritance pattern as well as metabolic and hormonal profiles. Patients were classified as having familial CPP if they had at least one first- or second-degree affected relative. Female relatives were considered affected when they had documented CPP or self-reported precocious menarche (≤9 years). Male relatives were considered affected when they had documented CPP or self-reported premature age at onset of pubertal changes (≤9 years), with addition of self-reported full puberty ≤13 years (including full facial shaving). Based on the clinical characterization, 36 (18%) out of 197 patients with CPP were classified as having multiple anomalies, such as neurocognitive abnormalities and/or other non-reproductive clinical features (>two manifestations). In order to undergo a comprehensive assessment, among these 36 patients, eight were also followed by a clinical geneticist, while another eight were also followed by a neuro-pediatrician. These 36 patients (25 sporadic cases, 11 familial cases from unrelated families; 32 girls, 4 boys) were selected for the genetic-molecular studies. The study protocol was approved by the local Ethics Committee. Written informed consent was obtained from all patients and/or their legal guardians.

Genetic studies

Genetic and epigenetic analysis were performed as illustrated in Fig. 1. First, all cases (n = 36) were investigated for (i) imprinting defects of *MKRN3* and *DLK1* through methylation-specific analysis and (ii) chromosomal abnormalities through chromosomal microarray analysis methodologies. Thereafter, a subset of patients (n = 9), who had no abnormal findings in methylation-specific and chromosomal microarray analysis, was submitted to whole-exome sequencing for screening coding variants. These patients (n = 9) were included for exome sequencing analysis for presenting (i) familial CPP (n = 7) or (ii) sporadic CPP associated with severe congenital malformations and neurocognitive abnormalities (n = 2). The genomic positions and the raw data of all experiments were aligned using the GRCh37/hg19 assembly of the human genome reference. All the methods employed have been previously described elsewhere, and conducted according to the standard protocol of the manufacturer (Villela *et al.*, 2017; Geoffron *et al.*, 2018; Vasques *et al.*, 2018).

Genomic DNA extraction

Genomic leukocyte DNA was extracted from whole blood samples from 36 patients and 46 relatives (15 maternal, 11 paternal, 14 from other first-degree relatives and 6 from second-degree relatives) using standard salting out procedure (Miller *et al.*, 1988).

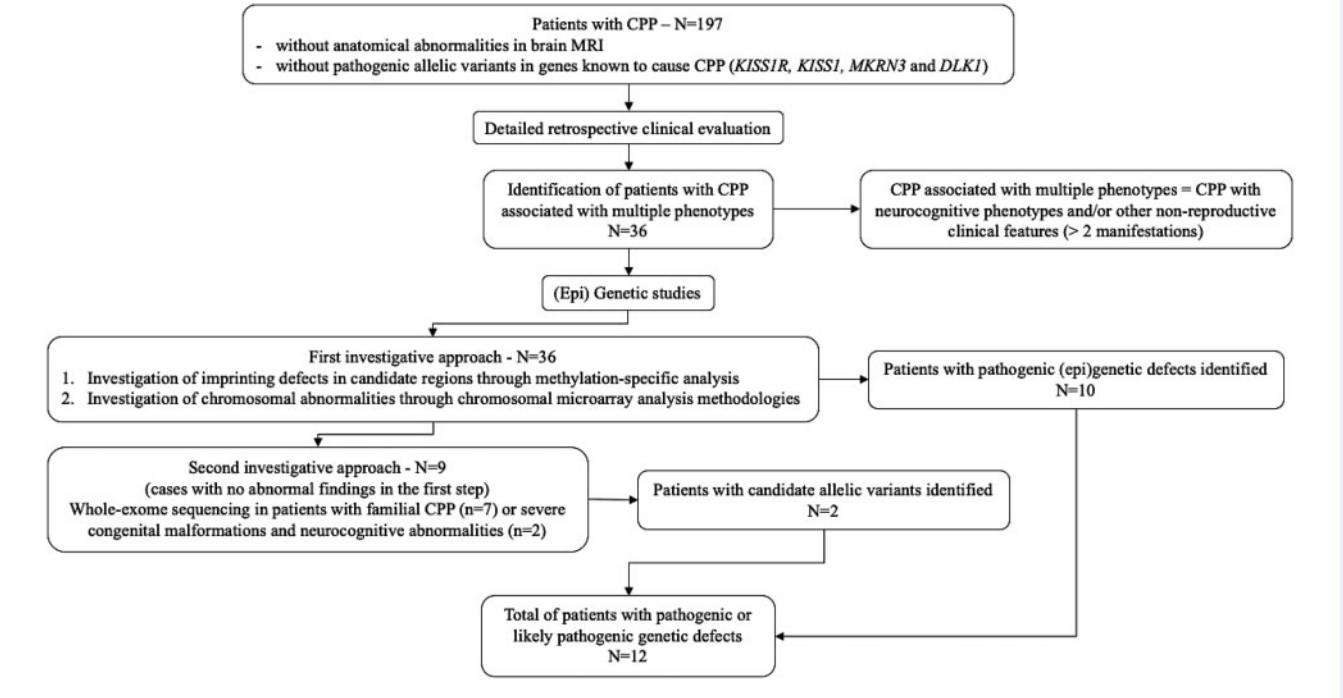

Figure 1. Flow chart showing the study design. CPP, central precocious puberty; N, number of patients.

DNA methylation analysis

All cases (n = 36) were investigated for imprinting defects at candidate regions. The methylation status at differentially methylated regions (DMR) of *MKRN3* (*MKRN3*:TSS-DMR) and *DLK1* (*DLK1/MEG3*:IG-DMR) were analysed separately. Briefly, leukocyte DNA was submitted to sodium bisulfite treatment (EZ DNA methylation lightning kit; Zymo Research, Orange, CA, USA) followed by TaqMan allele-specific methylated multiplex real-time quantitative PCR (ASMM-RTQ-PCR) (Geoffron *et al.*, 2018). The methylation status at each locus was reported as the methylation index (MI) (Geoffron *et al.*, 2018). The MI results were compared with 50 adult controls and 33 children controls with normal puberty. The MI was considered normal if it was within two standard deviations of the mean MI value for the control population. The MI was considered abnormal if outside this range.

Chromosomal microarray analysis

All cases (n = 36) were evaluated for copy number variants (CNVs) through chromosomal microarray analysis (Villela *et al.*, 2017). For 24 patients, single-nucleotide polymorphism array (SNP-array) was used for investigating CNV as well as uniparental disomy (UPD); comparative genomic hybridization microarray (aCGH) was performed for 12 cases. For CNV pathogenicity assessment, eight family members underwent chromosomal microarray for segregation analysis. SNP-array analysis was performed using the CytoSNP 850K BeadChip from Illumina (Illumina, CA, USA), while aCGH was carried out using a 180K oligonucleotide platform (Agilent Technologies, CA, USA). Detected CNVs were classified according to the American College of Medical Genetics and Genomics (ACMG) standards with five tiers of pathogenicity: pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, and benign (Riggs *et al.*, 2020). The main criteria for establishing the pathogenicity of CNVs were the following: (i) overlap with genomic coordinates for a known genomic disorder (ClinGen https://clinicalgenome.org; DECIPHER https://decipher.sanger.ac.uk); (ii) size, gene content (UCSC Genome Browser https://genome.ucsc.edu/cgi-bin/hgGateway) and type of the CNV; (iii) frequency of CNVs overlapping the segment in the general population (healthy individuals) according to both the Database of Genomic Variants and an in-house database from a cohort of 3000 individuals with the same ethnic background; and (iv) inheritance and segregation with the disorder.

Whole-exome sequencing analysis

Nine patients and 22 relatives underwent exome sequencing (Vasques *et al.*, 2018). The libraries were constructed with the SureSelect Target Enrichment system (Agilent Technologies, CA, USA). The sequences were generated in the Illumina HiSeq 2500 platform running on paired-end mode (Vasques *et al.*, 2018). Based on the family pedigree, the exome data were filtered for loss-of-function and non-synonymous variants present in a heterozygous state in the affected family members and not present in the unaffected family members. Exome sequencing data were screened for rare variants (minor allele frequency $\leq$ 0.01% in public and in-house database) located in exonic regions and consensus splice site sequences. Splice site sequences analysis were performed using dbscSNV and Human Splice Finder. The public database included gnomAD (Genome Aggregation Database) (including ExAC, Exome Aggregation Consortium), 1000Genomes (1000 Genomes Project Data) (Birney and Soranzo, 2015) and ABraOM (Online Archive of Brazilian Mutations) (Naslavsky *et al.*, 2017); while the in-house exome database (SELA Laboratory) included a local cohort of 523 unrelated individuals with distinct medical disorders with the same ethnic background. The variant filtration prioritized genes on the basis of their potential to be pathogenic: loss-of-function variants and variants predicted to be pathogenic by multiple *in silico* programs. Possible candidate sequence variants were classified according to the ACMG standards with five categories of pathogenicity: pathogenic, likely pathogenic, VUS, likely benign and benign (Richards *et al.*, 2015). The sequencing reads carrying candidate variants were inspected visually using the Integrative Genomics Viewer (IGV; Broad Institute, Cambridge, MA, USA) (Vasques *et al.*, 2018).

Results

Clinical characterization

The cohort of CPP associated with multiple anomalies (n = 36) was predominantly female (89%). At the first endocrinology evaluation, five patients presented short stature (height SDS < -2) and/or no bone age advancement, differently from the classical description of patients with CPP. The more prevalent clinical features included metabolic, growth and neurocognitive abnormalities. Less prevalent or rare clinical aspects included dysmorphic features and congenital anomalies (Table I).

Pathogenic or likely pathogenic genetic defects

Overall, pathogenic or likely pathogenic genetic defects were identified in 12 (33%) out of 36 patients with CPP associated with multiple anomalies. There were no leukocyte DNA methylation defects in *MKRN3*:TSS-DMR in any of the studied patients (Supplementary Table SI). Three patients with sporadic CPP presented with hypomethylation at *DLK1/MEG3*:IG-DMR (Patients 1, 2 and 3) . Nine patients presented chromosomal abnormalities in chromosomal microarrays: two UPD (Patients 1 and 2), three *de novo* deletions (Patients 4, 5 and 6), three inherited deletions (Patients 7, 8 and 9 from unrelated families) and one *de novo* duplication (Patient 10) (Supplementary Table SII). All these CNVs were absent in the in-house database and absent/rare in public database. Exome sequencing analysis revealed pathogenic or likely pathogenic candidate allelic variants in one sporadic female case (Patient 11) and one familial male case (Patient 12) (Supplementary Table SIII). These candidate variants were absent in the in-house and public databases. For the patients carrying pathogenic or likely pathogenic genetic defects, Table II shows the clinical and laboratory features of pubertal development and Table III shows the associated anomalies.

Genetic defects identified through chromosomal microarray analysis and methylation analysis

Patients 1, 2 and 3 presented with hypomethylation at *DLK1/MEG3*:IG-DMR with MI at 10%, 16% and 11%, respectively. As proposed by previous studies (Kagami *et al.*, 2017; Geoffron *et al.*, 2018), these three patients were subsequently submitted to the methylation analysis of *MEG3*:TSS-DMR, presenting also with hypomethylation at the secondary DMR of the 14q32.2 imprinted region, with MI at 1%,

Table I Clinical characterization of 36 patients with central precocious puberty associated with multiple anomalies.

Anthropometric and pubertal features	Mean ±SD (min–max)
Age at puberty onset, years	
Girls (n = 32)	5.7 ± 2.1 (0.4 to 8.0)
Boys (n = 4)	8.2 ± 0.5 (7.9 to 9.0)
At first visit	
Chronological age, years	7.8 ± 2.8 (1.7 to 14.9)
Height SDS	0.9 ± 1.3 (−2.1 to 3.9)
Target height SDS[a]	−0.3 ± 1.2 (−3.0 to 2.1)
Body mass index SDS	0.9 ± 1.1 (−1.0 to 3.4)
Bone age advancement, years[b]	1.7 ± 1.3 (1.4 to 4.1)
Tanner stage	2 to 4
Associated clinical features and conditions	**% (number of cases)**
Metabolic abnormalities	
High BMI at first visit	38.8% (14/36)
Overweight	22.2% (8/36)
Obesity	16.6% (6/36)
Acanthosis and/or hyperinsulinemia	16.6% (6/36)
Polycystic ovary syndrome (as a young adult)	15% (4/26)
Early onset type 2 diabetes	2.7% (1/36)
Growth abnormalities	
Born small for gestational age	33% (9/27)
Growth retardation or absence of advanced bone age at initial diagnosis of CPP	13.8% (5/36)
Short stature at adult height (in patients treated with aGnRH)[c]	14% (3/21)
Treatment with rhGH (concomitant with aGnRH)	14% (5/36)
Disproportional stature	11% (4/36)
Neurocognitive abnormalities	
Learning difficulties or intellectual disability	33% (12/36)
Attention deficit and hyperactivity disorder	8% (3/36)
Autism spectrum disorder	2.7% (1/36)
Motor delay and/or speech delay	25% (9/36)
Less prevalent or rare clinical features	
Axial skeletal deformities	16.6% (6/36)
Clinodactyly	16.6% (6/36)
High palate	14% (5/36)
Irregular teeth	14% (5/36)
Premature delivery	15% (4/27)
Ear anomalies	11% (4/36)
Non-autoimmune hypothyroidism	8% (3/36)
Autoimmune hypothyroidism	5.5% (2/36)
Prominent forehead	8% (3/36)
Hypertelorism	8% (3/36)
Small hands and/or feet	8% (3/36)
Chronic constipation	8% (3/36)
Madelung deformity	5.5% (2/36)
Born large for gestational age	7.4% (2/27)
Tall stature or height > 2 SD above the target height	9.5% (2/21)
Valvar aortic stenosis	5.5% (2/36)
Strabismus	5.5% (2/36)
Imperforate anus, triangular face, abnormal gait, palpebral ptosis, ventricular arrhythmia, blue sclera, congenital clubfoot, gingival hyperplasia, high blood pressure, hearing loss	Each one in only 1 case

aGnRH, gonadotropin releasing hormone analog; CPP, central precocious puberty; rhGH, recombinant human growth hormone; SDS, standard deviation score.

[a]The target height was calculated by the arithmetic mean of parental height (paternal height plus maternal height divided by 2) with the addition or subtraction of 6.5 cm for boys and girls, respectively.

[b]Bone age advancement was expressed as bone age minus chronological age (BA−CA).

[c]Despite the treatment with GnRH analog, three patients presented short stature at adult height (adult height ≤ −2.0 SDS). In addition, these three patients presented adult height below the normal range of the target height, as well as below their parental heights.

15% and 4%, respectively. SNP-array in Patients 1 and 2 identified a maternal UPD at chromosome 14 (UPD(14)mat). Meanwhile, Patient 3 had normal chromosomal microarray analysis, as well as normal microsatellites analysis, excluding an UPD(14)mat. Due to the method limits of detection, chromosomal microarray analysis could have missed a smaller CNV encompassing the *DLK1* region in Patient 3. Sequentially, two MLPA analysis were performed in Patient 3: (i) the MS-MLPA probemix UPD14/UPD7, containing probes for two exons of *DLK1* gene, as well as probes for the promoter region and for two exons of the *MEG3* gene and (ii) a customized MLPA for *DLK1* gene (containing probes for all 5 exons of the gene). No copy number changes were identified in both experiments, indicating a mechanism of epimutation at *DLK1/MEG3*:IG-DMR (14q32.2 epimutation). Patient 1 was a boy firstly referred for presenting mild growth retardation, and he developed CPP and overweight during follow-up. Meanwhile, Patients 2 and 3 were both girls, firstly referred for presenting menarche at 8.7 years and at 7.9 years, respectively. At the endocrinology evaluation, other clinical findings were noticed in these patients, such as being born SGA, prominent forehead, small hands/feet, truncal overweight/obesity and early onset type 2 diabetes in Patient 3.

Patients 4, 5 and 6 carried *de novo* 7q11.23 deletions which represent a contiguous gene deletion syndrome known as Williams–Beuren syndrome (OMIM 194050). They presented characteristic clinical findings of this syndrome, such as typical dysmorphic face, intellectual disability and hypersociability, allowing a syndromic diagnosis early in life at a Genetic unit. As they had a history of premature sexual development, they were enrolled in the present study. The aCGH results precisely delineated the CNVs size, gene content and breakpoints. Patients 4 and 5 presented hemizygous 1.5 Mb deletions affecting the same 25 OMIM genes, whereas Patient 6 had a 1.37 Mb deletion

Table II Clinical and laboratory features of pubertal development of patients (and their affected relatives) with CPP carrying pathogenic or likely pathogenic genetic defects.

ID	Genetic defects[a]	CPP inheritance	Sex	Initial pubertal signs and/or menarche		At the CPP diagnosis					LH (IU/L)[a]		T (ng/dl) in boys and E2 (pg/ml) in girls	Age of treatment with GnRHa (years)	
				Condition	CA (years)	CA (years)	Tanner stage	Height SDS	BMI SDS	BA (years)	Basal	After GnRH-stimulation		At the onset	At the end
1	UPD(14)mat (hypomethylation at *DLK1/MEG3*:IG-DMR)	Sporadic	M	Testicular enlargement	7.9	7.9	G2	−1.8	2.0	6.5	2.3	–	78	7.9	12.8
2	UPD(14)mat (hypomethylation at *DLK1/MEG3*: IG-DMR)	Sporadic	F	Thelarche Menarche	6.0 8.7	9.7	B5	0.3	1.5	13	8.6	–	43.8	–	–
3	14q32.2 epimutation (hypomethylation at *DLK1/MEG3*:IG-DMR)	Sporadic	F	Thelarche Menarche	7.0 7.9	8.0	B5	0.7	2.3	12	6.6	–	17	8.0	11.8
4	1.5 Mb 7q11.23 deletion	Sporadic	M	Testicular enlargement	9.0	–	–	–	–	–	–	–	–	–	–
5	1.5 Mb 7q11.23 deletion	Sporadic	F	Thelarche Menarche	7.5 9.0	7.5	B2	0.9	0.1	–	1.2	–	29	–	–
6	1.37 Mb 7q11.23 deletion	Sporadic	F	Thelarche	7.5	7.5	B2	0.0	−0.8	8.9	0.3	6.5	–	7.5	In use
7	605 Kb Xp22.33 deletion	Index	F	Thelarche	6.2	7.8	B3	−1.9	1.4	5.9	3.5	30	21.1	7.8	13.6
		Brother	M	Testicular enlargement	10.0	10.0	G2	−1.4	1.4	7.5	0.94	–	25	10.0	11.5
		Father	M	Puberty onset Shaving	9.0 12.0	–	–	–	–	–	–	–	–	–	–
		Paternal aunt	F	Thelarche Menarche	6.0 7.0	–	–	–	–	–	–	–	–	–	–
8	731 Kb Xp22.33 deletion	Index	F	Pubarche Thelarche	5.3 7.5	7.8	B2	0.6	2.5	11.5	1.4	–	<15	7.8	10.5
		Sister	F	Thelarche	4.5	15.4	B5	−2.5	0.5	–	–	–	–	7.5	11.5
		Father	M	Not available	–	–	–	–	–	–	–	–	–	–	–
		Paternal aunt	F	Menarche	9.0	–	–	–	–	–	–	–	–	–	–
		Paternal aunt	F	Menarche	9.0	–	–	–	–	–	–	–	–	–	–
9	220 Kb Xp22.33 deletion	Index	F	Thelarche Menarche	7.9 8.8	8.8	B3	2.0	1.5	11.0	2.6	–	–	8.8	11.7
		Sister	F	Thelarche	9.4	9.7	B2	0.9	0.7	10.0	–	–	–	10.1	12.5
10	425 Kb 1p31.3 duplication	Sporadic	F	Thelarche	1.4	2.3	B4	1.5	−0.6	2.5	0.1	8.2	14	2.8	10.3
11	1) *TNRC6B* frameshift deletion	Sporadic	F	Thelarche	1.6	2.8	B3	1.0	−1.0	3.6	0.2	9.1	15	3.2	10.5

(continued)

Table II Continued

ID	Genetic defects[a]	CPP inheritance	Sex	Initial pubertal signs and/or menarche		At the CPP diagnosis					LH (IU/L)[a]		T (ng/dl) in boys and E2 (pg/ml) in girls	Age of treatment with GnRHa (years)	
				Condition	CA (years)	CA (years)	Tanner stage	Height SDS	BMI SDS	BA (years)	Basal	After GnRH-stimulation		At the onset	At the end
	2) *ARELI* frameshift insertion														
12	1) *UGT2B4* missense	Index	M	Testicular enlargement	8.0	9.4	B3	2.7	0.8	–	6.2	–	440	9.4	11.5
	2) *MKKS* frameshift deletion	Mother	F	Menarche	9.0	–	–	–	–	–	–	–	–	–	–
		Maternal half-sister	F	Menarche	8.0	–	–	–	–	–	–	–	–	–	–
		Maternal aunt	F	Menarche	8.0	–	–	–	–	–	–	–	–	–	–

B, breast stage; CA, chronological age; CPP, central precocious puberty; E2, estradiol; F, female; G, genital stage; M, male; SDS, standard deviation score; T, testosterone.
[a]The hormonal diagnosis of central puberty was based on basal and/or GnRH-stimulated LH levels within the pubertal range. In Patients 1, 2, 3, 5, 6, 10 and 12, LH levels were measured by electrochemiluminometric assay. In this setting, diagnostic values for basal LH were ≥0.3 IU/l in both sexes, while diagnostic values for GnRH-stimulated LH were >5.0 IU/l in both sexes. In Patients 7, 8, 9 and 11, LH levels were measured by immunofluorometric assay. In this setting, diagnostic values for basal LH were >0.6 IU/l in both sexes, while diagnostic values for GnRH-stimulated LH were >6.9 IU/l for girls and >9.6 IU/l for boys.
[a]Patients 1 to 6 presented genetic defects in *loci* known to be associated with CPP; whereas Patients 7 to 12 presented genetic defects in candidate genes or regions to the involvement with CPP.

involving 23 OMIM genes. The deletions overlapped with each other affecting the same gene content (Supplementary Table SIV).

Patients 7, 8 and 9 presented inherited Xp22.33 deletions affecting the pseudo-autosomal region 1 (PAR-1) and including the deletion of short stature homeobox (*SHOX*) gene (OMIM 312865). The three patients had similar clinical presentations, being firstly referred for presenting precocious puberty. Detailed clinical evaluation evidenced growth abnormalities resembling *SHOX* defects manifestations, such as disproportional short stature or Madelung deformity. In families 7 and 8, pedigree analysis showed a familial history of early sexual development with precocious puberty and *SHOX* abnormalities co-segregating in a dominant pattern of inheritance (Supplementary Fig. S1). Patient 9 had a younger sister with early puberty and the same *SHOX* abnormality. The aCGH provided a detailed overview of the CNVs aspects. In Patients 7, 8 and 9, the deletions encompassed *SHOX* and conserved non-coding elements, known as enhancers (Supplementary Table SIV). Patients 7 and 8 harbored larger inherited deletions (affecting 4 additional genes), which segregated with the puberty disorder. Attempting to delineate a Xp22.33 segment potentially involved in CPP, we also performed the aCGH of five unrelated patients with *SHOX* growth abnormalities but with normal puberty. The comparison of chromosomal microarray analysis of patients with CPP and patients with normal puberty showed an overlap of the genomic segments of the deletions (Supplementary Fig. S2). It is of note that a rare Xp22.33 duplication, encompassing a regulatory region without coding genes, which was classified as VUS, was identified in a girl (Patient 13) with sporadic CPP (Supplementary Table SV).

Patient 10 presented with sporadic CPP, ventricular arrhythmia and learning difficulties. She was the only child of non-consanguineous parents. SNP-array identified a pathogenic *de novo* 1p31.3 duplication of 425 Kb affecting only the nuclear factor 1/A (*NFIA*) gene (OMIM 600727).

Genetic defects identified through exome sequencing

Patient 11 was a girl with sporadic CPP, neurocognitive delay, dysmorphic features and imperforate anus. She was the only child of non-consanguineous parents. Exome sequencing analysis through a trio-based approach identified two rare loss-of-function variants in a dominant *de novo* pattern: (i) a pathogenic frameshift deletion (p.Gly665Leufs*35) in the trinucleotide repeat-containing gene 6B (*TNRC6B*) gene (OMIM 610740) and (ii) a likely pathogenic frameshift insertion (p.Ser229Phefs*3) in the apoptosis-resistant E3 ubiquitin protein ligase 1 (*ARELI*) gene (OMIM 615380).

Patient 12 was a boy with CPP, autism spectrum disorder, tall stature and dysmorphic features. He presented with a maternally inherited CPP following a dominant inheritance pattern, where his mother, maternal half-sister and maternal aunt had a history of precocious menarche (Supplementary Fig. S1). Exome sequencing analysis of six family members (four affected and two unaffected) identified two rare candidate variants segregating with the puberty disorder: (i) a missense substitution (p.Pro267Leu) in the uridine diphosphate glycosyltransferase 2 family member 4 (*UGT2B4*) gene (OMIM 600067), classified as a VUS and (ii) a frameshift deletion (p.Phe144Leufs*14) in the McKusick-Kaufman syndrome (*MKKS*) gene (OMIM 604896). The *UGT2B4* variant was predicted to be disease-causing in 11 in-silico models (Polyphen2, Mutation Assessor, Mutation Taster, Sift, Provean, MetaSVM, FATHMM-KL, DANN, EIGEN, PrimateAI and Revel). This

Table III Associated anomalies in patients (and their affected relatives) carrying pathogenic or likely pathogenic genetic defects.

ID	Genetic defect [a]	CPP inheritance	Metabolic abnormalities	Growth abnormalities	Neurocognitive abnormalities	Congenital anomalies, dysmorphic features, and other anomalies	Associated therapies
1	UPD(14)mat (hypomethylation at *DLK1/MEG3*:IG-DMR)	Sporadic	Perinatal hypoglycaemia Obesity	Premature delivery Post-natal growth retardation	Mild speech delay Mild motor delay	Prominent forehead, almond shaped eyes, irregular teeth, high palate, small hands and feet, clinodactyly	rhGH
2	UPD(14)mat (hypomethylation at *DLK1/MEG3*:IG-DMR)	Sporadic	Overweight	SGA for weight and length (BW SDS −4.6 and BL SDS −4.7) Short stature	Mild speech delay Mild motor delay Learning difficulties	Prominent forehead, irregular teeth, high palate, small hands and feet, clinodactyly	
3	14q32.2 epimutation (hypomethylation at *DLK1/MEG3*:IG-DMR)	Sporadic	Obesity, acanthosis Dyslipidemia Early onset type 2 diabetes	Limit SGA for weight (BW SDS −2.0 and BL SDS −1.5) Short stature	Mild speech delay Mild motor delay	Prominent forehead, almond shaped eyes, almond shaped eyes, small hands and feet, clinodactyly	Metformin Gliclazide Pioglitazone
4	1.5 Mb 7q11.23 deletion	Sporadic	NA	Short stature	Intellectual disability Developmental delay	Typical dysmorphic face Pulmonary stenosis Primary hypothyroidism	–
5	1.5 Mb 7q11.23 deletion	Sporadic	Obesity at adulthood	–	Intellectual disability Hypersociability	Typical dysmorphic face Chronic constipation Ligament laxity	–
6	1.37 Mb 7q11.23 deletion	Sporadic	–	SGA for length (BW SDS −1.7 and BL SDS −2.1) Short stature	Intellectual disability Hypersociability	Typical dysmorphic face Supravalvar aortic stenosis	–
7	605 Kb Xp22.33 deletion	Index	Overweight	Disproportional stature (H SDS −1.6 and SH/H SDS 3.5) Mild Madelung deformity	–	Blue sclera	rhGH
		Brother	–	SGA for length (BW SDS −0.7 and BL SDS −4.2) Disproportional short stature (H SDS −2.5 and SH/H SDS 4.3)	–	–	–
		Father	Obesity at adulthood	Short stature (H SDS −4.1)	–	–	–
		Paternal aunt	Obesity at adulthood	Short stature (H SDS −4.7)	–	–	–
8	731 Kb Xp22.33 deletion	Index	Obesity Hyperinsulinemia	SGA for length (BW SDS 0.6 and BL SDS −2.5) Disproportional stature (H SDS 0.2 and SH/H SDS 2.0) Mild Madelung deformity	–	–	rhGH Metformin
		Sister	–	Disproportional short stature (H SDS −2.6 and SH/H SDS 2.3) Madelung deformity	–	Hearing loss (recurrent otitis in childhood)	–
		Father	–	Disproportional stature (H SDS −1.4 and SH/H SDS 2.2)	–	–	–
		Paternal aunt	–	Madelung deformity	–	–	–
		Paternal aunt	–	Madelung deformity	–	–	–
9	220 Kb Xp22.33 deletion	Index	Overweight	Limb shortening	–	–	rhGH Metformin
		Sister	–	Limb shortening	–	–	

(continued)

Table III Continued

ID	Genetic defect [a]	CPP inheritance	Metabolic abnormalities	Growth abnormalities	Neurocognitive abnormalities	Congenital anomalies, dysmorphic features, and other anomalies	Associated therapies
10	425 Kb 1p31.3 duplication	Sporadic		Premature delivery	Learning difficulties	Hypertelorism Ventricular arrhythmia	Atenolol
11	1) *TNRC6B* frameshift deletion 2) *AREL1* frameshift insertion	Sporadic		SGA for weight and length (BW SDS −2.0 and BL SDS −3.1)	Intellectual disability (total IQ 80, lower limit) Attention deficit disorder	Strabismus, triangular face, low-set posteriorly rotated ears, clinodactyly Imperforate anus	Speech therapy Methyl-phenidate
12	1) *UGT2B4* missense 2) *MKKS* frameshift deletion	Index	–	LGA for weight (BW SDS 2.6 BL SDS 1.2) Tall stature (H SDS 2.9)	Autism disorder Abnormal gait	Bilateral palpebral ptosis, high palate, irregular teeth	Aripiprazole Fluoxetine Pericyazine
		Mother	Obesity at adulthood	Normal height despite no treatment for CPP (H SDS −0.5)	–	High blood pressure	–
		Maternal half-sister	Overweight at adolescence	Normal height despite no treatment for CPP (H SDS 0.9)	–	–	–
		Maternal aunt	–	–	–	–	–

BL SDS, birth length standard deviation score (calculated by Usher & McLean method); BW SDS, birth weight standard deviation score (calculated by Usher & McLean method); H SDS, height standard deviation score; IQ, intelligence quotient; LGA, large for gestational age; NA, not available; rhGH, recombinant human growth hormone; SGA, small for gestational age; SH/H SDS, sitting height/height ratio standard deviation score; UPD(14)mat, maternal uniparental disomy of chromosome 14.

[a]Patients 1 to 6 presented genetic defects in *loci* known to be associated with CPP; whereas Patients 7 to 12 presented genetic defects in candidate genes or regions to the involvement with CPP.

gene encodes for an enzyme involved in the glucuronidation of androgens in the liver (Turgeon *et al.*, 2001; Barre *et al.*, 2007). Thus, we performed a 24 h-urinary steroid metabolome analysis in six family members (four affected and two unaffected). The metabolome revealed normal androgen and glucocorticoid excretion patterns in the affected family members with no difference compared to unaffected members (Supplementary Table SVI). Regarding the *MKKS* frameshift deletion, it was speculated as a candidate due to the presence of a null variant segregating with precocious puberty in the family. Indeed, using ACMG criteria for analysis, the variant was categorized as likely pathogenic. Considering the CPP maternal inheritance in this male patient, we also performed an analysis of variants in the mitochondrial DNA using the exome data. However, no pathogenic variant was identified.

Discussion

In the present study, 36 (18%) out of 197 unrelated patients with CPP presented multiple anomalies, suggesting the involvement of genetic factors in these cases. We identified pathogenic or likely pathogenic genetic defects in 12 (33%) of them. CNVs were the most prevalent genetic defect (7 cases), followed by epigenetic abnormalities (3 cases) and identification of rare sequence variants (2 cases) in candidate genes using exome sequencing. Notably, the most frequent associated clinical findings in this particular CPP group were metabolic, growth and neurocognitive anomalies, pointing out to potential disruptions in mechanisms regulating factors of human development (Wright *et al.*, 2018). Some clinical manifestations in patients with CPP should indicate medical attention for investigating genetic causes of CPP, such as short stature at presentation or after GnRH analog treatment, being born SGA, learning difficulties/intellectual disabilities, motor and/or speech delay, rare congenital malformations, and early onset metabolic abnormalities (particularly type 2 diabetes mellitus).

Evidence has demonstrated that puberty is highly sensitive to metabolic and nutritional factors (Vazquez *et al.*, 2018). Indeed, epidemiologic studies have shown an association between earlier puberty and overweight/obesity in girls, which has not been observed similarly in boys (Soriano-Guillén and Argente, 2019). It has been postulated that body fat may modulate pubertal timing, acting through distinct permissive or inhibiting factors (Kaplowitz, 2008). In contrast, high BMI is particularly relevant when in association with insulin resistance conditions and central fat distribution, characterizing metabolic syndrome and contributing to a syndromic diagnosis. It is of note that 14 (39%) of the 36 selected patients in the present study presented with high BMI, which could have influenced pubertal timing. However, high BMI was considered as an inclusion criterium in the cohort when in association with other abnormalities, as this picture could more likely indicate a genetic disorder. Likewise, three (8%) of the 36 selected patients had attention deficit and hyperactivity disorder (ADHD). Besides ADHD and CPP, these three patients presented with other clinical abnormalities such as intellectual disability, skeletal anomalies and/or growth deficit. In fact, isolated ADHD does not seem to be related with CPP. In contrast, it may be a clinical feature of genetic conditions, when in association with other abnormalities.

Three patients (Patients 1, 2 and 3) with sporadic CPP associated with short stature presented with hypomethylation at *DLK1/MEG3*:IG-DMR, a mechanism implicated in the lack of expression of the paternal copy of *DLK1* gene. This epigenetic mechanism has been associated with Temple syndrome (OMIM 616222), a rare imprinting disorder, characterized by CPP in 80–90% of cases (Kagami *et al.*, 2017; Geoffron *et al.*, 2018). It has been described that clinical features in patients with Temple syndrome may be heterogeneous and non-specific, leading to a difficulty of the diagnosis in early childhood or adulthood (Ioannides *et al.*, 2014). Recently, Kagami *et al.* (2017) has proposed clinical indications for the genetic investigation of Temple syndrome; precocious puberty was included as an indicator. Notably, paternally inherited loss-of-function variants of *DLK1* have been identified as a cause of non-syndromic CPP (Dauber *et al.*, 2017; Gomes *et al.*, 2019; Montenegro *et al.*, 2020). *DLK1* is a noncanonical ligand in the Delta-Notch signaling pathway, known to play a role in several cell types, mostly in inhibiting adipocyte differentiation. Gomes *et al.* (2019) demonstrated that patients with CPP due to *DLK1* loss-of-function variants more often developed metabolic abnormalities (obesity, hyperlipidemia and early onset glucose intolerance/type 2 diabetes) in adult life, when compared to patients with idiopathic CPP, suggesting that *DLK1* could represent a novel link between reproduction and metabolism. Likewise, Patients 1, 2 and 3 with hypomethylation at *DLK1/MEG3*:IG-DMR also presented CPP/precocious menarche, overweight/obesity, dyslipidemia and type 2 diabetes mellitus. Therefore, we believe that *DLK1* deficiency is probably the leading cause of CPP in patients with Temple syndrome (Dauber *et al.*, 2017).

The Williams–Beuren syndrome is a multi-system disorder caused by deletion of the 7q11.23 chromosome region (Pober, 2010). The condition is considered a contiguous gene syndrome and is known by a clinical heterogeneity among patients with deletions of the same size and breakpoints (Pober, 2010). CPP (or precocious menarche) has been described in about 10% (3–18%) of Williams–Beuren syndrome cases, depending on the case series (Partsch *et al.*, 2002; Pober, 2010). Indeed, some studies have shown that up to 50% of patients with Williams–Beuren syndrome may present an early puberty, which is defined by the age at puberty onset at the lower limit of the normal range (Kim *et al.*, 2016). In addition, girls with Williams–Beuren syndrome, on average, present menarche 2 years earlier than control girls (Pober, 2010). Interestingly, Perry *et al.* (2014) identified an enriched signal for association with age at menarche in the region of Williams–Beuren syndrome (*GTF2I* gene *locus*) in a robust genome-wide association study (GWAS).

SHOX deficiency is a frequent genetic growth disorder with clinical variability among distinct affected families. Defects in one copy of the gene are mainly characterized by isolated (disproportional) short stature or Léri–Weill dyschondrosteosis (OMIM 127300; mesomelic short stature and Madelung deformity) (Marchini *et al.*, 2016). Three patients with CPP (Patients 7, 8, and 9) had Xp22.33 deletions mapped within the PAR-1 and involving *SHOX* gene. Notably, CPP is not a condition typically related with *SHOX* gene alterations itself (Scalco *et al.*, 2010). These three patients belonged to three distinct families with CPP and disproportional (short) stature with or without Madelung deformity. *SHOX* defects have been described mainly as CNVs (~80%), with deletions involving the gene completely or partially (Marchini *et al.*, 2016). To date, 24 genes have been assigned to the human PAR-1, but *SHOX* is the only known disease gene within the region. The PAR-1 genes are known to exhibit 'pseudo-autosomal' rather than X-linked inheritance (Blaschke and Rappold, 2006). It is established that PAR-1

has a high recombination frequency (crossover rate) during meiosis in males, resulting in the possibility of transference of gene content within the PAR-1 from the X chromosome to the Y chromosome and vice-versa (Kant et al., 2011). This phenomenon explains the inheritance of the deletion in the family of Patient 7, in which her affected brother inherited the Xp22.33 deletion from their affected father (Supplementary Fig. S1). The potential contribution to pubertal development of genes in X chromosome has been proposed by a study of methylome profiling in girls with normal and precocious puberty (Bessa et al., 2018), as well as by case reports of premature sexual development in patients with X chromosome structural variants (Giorda et al., 2009; Bessa et al., 2018). In the present study, the aCGH could not point out a specific gene implicated in the pubertal disorder within the Xp segment. However, we hypothesized that the disruption of a still unknown genetic factor in cis in the X chromosome could probably be involved in the CPP of patients with Xp22.33 variants in this study.

Patient 10, who had sporadic CPP, ventricular arrhythmia and learning difficulties, presented a very rare *de novo* variant that partially duplicated the sequence of the *NFIA* gene. This gene encodes a protein member of the nuclear factor I family that functions as a transcription factor (Scavuzzo et al., 2018) and has hypothalamic expression (https://www.gtexportal.org/home/gene/NFIA). Recently, Scavuzzo et al. (2018) showed that *NFIA* regulates pancreatic endocrine progenitor induction and that NFIA-deficient cells have a gain in Notch signaling and cell fate defects. Notably, *DLK1* is a non-canonical ligand in the Notch pathway signaling (Dauber et al., 2017). We speculated that the partial duplication of *NFIA* could have led to a NFIA gain-of-function effect, leading to a loss in Notch signaling, which could manifest as an early activation of the HPG axis and CPP, similar to *DLK1* deficiency conditions.

In Patient 11, exome analysis revealed two candidate *de novo* allelic variants in distinct genes, *TNRC6B* and *AREL1*, both with hypothalamic expression (https://gtexportal.org/home/gene/TNRC6B and https://gtexportal.org/home/gene/AREL1). *TNRC6B* plays a role in RNA-mediated gene silencing, regulating translational inhibition. Through GeneMatcher (Sobreira et al., 2015), Patient 11 was included in a collaboration work phenotyping 16 patients with *TNRC6B* loss-of-function variants (Granadillo et al., 2020). All patients presented neurocognitive abnormalities, as well as variable dysmorphic features and congenital anomalies; however, CPP was reported only in Patient 11, suggesting that the *TNRC6B* variants were strongly associated with the neurocognitive and dysmorphic abnormalities, but not with CPP. Nevertheless, it is possible that the full spectrum of clinical phenotypes of *TNRC6B* loss-of-function variants has not been delineated yet, as there are few patients reported. *AREL1* encodes an E3 ubiquitin ligase that inhibits apoptosis by ubiquitinating and degrading proapoptotic proteins (Kim et al., 2013), acting similarly to the pubertal regulating gene *MKRN3* (Abreu et al., 2013). *AREL1* has not yet been associated with human disorders; however, GWAS showed SNPs effects of this gene for teat number in pigs, indicating its possible association with reproductive abnormalities (Tan et al., 2017). Thus, the association of pubertal disorder with one of these two candidate genes is currently a speculation to be better delineated.

Patient 12, a boy with CPP, autism spectrum disorder, tall stature and dysmorphic features, harbored two rare candidate allelic variants in distinct genes, *UGT2B4* and *MKKS*, both segregating with premature sexual development in family members. Despite being predicted as disease-causing by *in silico* models, the *UGT2B4* variant was characterized as VUS for the gene not showing disease associations so far. This gene encodes for an enzyme involved in the glucuronidation of androgens (Barre et al., 2007), and GWAS analysis previously demonstrated an association with age at menarche for this locus in specific populations (Yermachenko and Dvornyk, 2016). We hypothesized that reduced systemic androgen clearance due to impaired glucuronidation could cause androgen excess, which might trigger premature reactivation of the HPG axis resulting in CPP. However, the urinary steroid metabolome analysis showed normal results in affected family members. This suggests that the *UGT2B4* variant is not directly involved in steroid metabolism and its significance remains to be elucidated. Regarding the *MKKS* gene, it encodes a protein which has an important role in cytokinesis and hypothalamic expression (https://gtexportal.org/home/gene/MKKS). Despite being suggested as a possible candidate, it is of note that *MKKS* heterozygous variant have not yet been associated with known diseases, and nor has the gene been associated with pubertal timing.

It is of note that, from the total of 36 patients with CPP associated with multiple anomalies, 27 have been properly treated with long-acting GnRH analogs for a mean time of 3.2 years, reaching adequate clinical and laboratory control. These initial data suggest that in patients with CPP and multiple anomalies, the treatment with GnRH analogs is effective (Latronico et al., 2016). This positive experience is in line with few studies reporting good responses with the use of GnRH analogs in syndromic patients (Partsch et al., 2002; Scalco et al., 2010; Geoffron et al., 2018).

In conclusion, the results have highlighted the relevance of an integrative clinical-genetic approach in the elucidation of genetic and epigenetic mechanisms involved in reproductive abnormalities. The accurate molecular diagnosis may enhance our abilities in the management, treatment and the long-term follow-up for each patient, as well as in the reproductive counseling for each family. The loss of imprinting of *DLK1* locus was identified as a probable mechanism of CPP. In addition, chromosome regions 7q11.23 and Xp22.33 represented candidate loci potentially involved in the pubertal timing disorder, to be considered for future studies. Altogether, these findings provided insights into the genetics of human pubertal control.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Authors' roles

Conception of the study: A.P.M.C., A.C.L. Design of the study: A.P.M.C., A.C.V.K., B.B.M., I.N., V.N.B., A.C.L. Acquisition of data: A.P.M.C., R.S.H., C.A.K., F.Z., A.A.L.J., B.B.M., V.N.B., A.C.L.

Performance of experiments: A.P.M.C., L.R.M., S.C., V.S., M.L.S., R.S.H., L.C.G., M.F.A.F. Analysis of data: A.P.M.C., A.C.V.K., L.R.M., S.C., C.R., V.S., M.L.S., L.S., R.S.H., C.A.K., F.Z., J.I., L.C.G., W.A., M.F.A.F., A.A.L.J., I.N., A.C.L. Interpretation of data: A.P.M.C., A.C.V.K., L.R.M., S.C., C.R., J.I., W.A., M.F.A.F., A.A.L.J., B.B.M., I.N., V.N.B., A.C.L. Draft of the manuscript: A.P.M.C. Critical revision of the manuscript for important content: A.C.V.K., L.S., R.S.H., F.Z., J.I., W.A., A.A.L.J.., B.B.M., I.N., V.N.B., A.C.L. Final edition of the manuscript: A.P.M.C., V.N.B., A.C.L.

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Conflict of interest

The authors have nothing to disclose.

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