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CASE REPORT *Reproductive genetics*

A novel MKRN3 missense mutation causing familial precocious puberty

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ABSTRACT: Central precocious puberty may be familial in about a quarter of the idiopathic cases. However, little is known about the genetic causes responsible for the disorder. In this report we describe a family with central precocious puberty associated with a mutation in the *makorin RING-finger protein 3 (MKRN3)* gene. A novel missense mutation (p.H420Q) in the imprinted MKRN3 gene was identified in the four affected siblings, in their unaffected father and in his affected mother. An *in silico* mutant *MKRN3* model predicts that the mutation p.H420Q leads to reduced zinc binding and, subsequently, impaired RNA binding. These findings support the fundamental role of the *MKRN3* protein in determining pubertal timing.

Key words: central precocious puberty / maternal imprinting / zinc finger / makorin RING-finger protein 3

Introduction

The timing of puberty is influenced by stimulating and restraining factors, many of which are still unknown. The study of pathological states of early and delayed puberty has provided valuable insight into those factors regulating GnRH activity.

Central precocious puberty (CPP), caused by early activation of pulsatile GnRH secretion, may result from hypothalamic tumors or lesions but in most cases is idiopathic. We have previously shown that idiopathic precocious puberty was familial in as many as 27.5% of cases and segregation analysis suggested autosomal dominant transmission with incomplete, sex-dependent penetrance (de Vries *et al.*, 2004). To date, mutations associated with CPP have been identified in the genes encoding kisspeptin 1 and its receptor (*KISS1* and *KISS1R*, respectively) (Teles *et al.*, 2008; Silveira *et al.*, 2010), and in the makorin RING-finger protein 3 (MKRN3) gene (Abreu et al. 2013).

The first mutation in the *MKRN3* gene was described in 2013 (Abreu *et al.*, 2013). Up to now, mutations have been described in eight families with familial CPP (Abreu *et al.*, 2013; Schreiner *et al.*, 2014; Settas *et al.*, 2014) and in eight girls with apparently sporadic CPP (Macedo *et al.*, 2014).

In this study we report on a novel missense mutation in the *MKRN3* gene found in four siblings who presented with CPP, their unaffected father and his affected mother.

Materials and Methods

Subjects

Four otherwise healthy siblings presented with idiopathic CPP (Fig. 1A). All had normal psychomotor development with high scholastic performance at school or in kindergarten. The non-consanguineous parents, of mixed Ashkenazi-Sephardic Jewish origin, are healthy and in both parents pubertal development was normal: maternal age at menarche was 12 years, and paternal age at first full-facial shaving was 17 years. The paternal grandmother had her menarche between 9 and 10 years of age and the paternal grandfather reported normal puberty. The two non-married paternal sisters were reported to have had normal puberty. None of the family members presented with fertility problems or other non-reproductive phenotypic features.

Pubertal stage was evaluated according to Marshall and Tanner (1969) and bone age according to Greulich and Pyle (1959). Growth velocity assessment at diagnosis was based on two height measurements taken at least 6 months apart.

All patients underwent a GnRH stimulation test. Serum basal LH $>$ 0.1 U/l, peak GnRH-stimulated LH $>$ 5.0 U/l, basal estradiol $>$3 pmol/l and basal testosterone $>$0.4 nmol/l were considered to be pubertal levels (Neely *et al.*, 1995; de Vries *et al.*, 2006).

Pituitary imaging was performed by magnetic resonance imaging (MRI). Transabdominal pelvic ultrasound scans were performed in all three sisters as previously described (de Vries *et al.*, 2006).

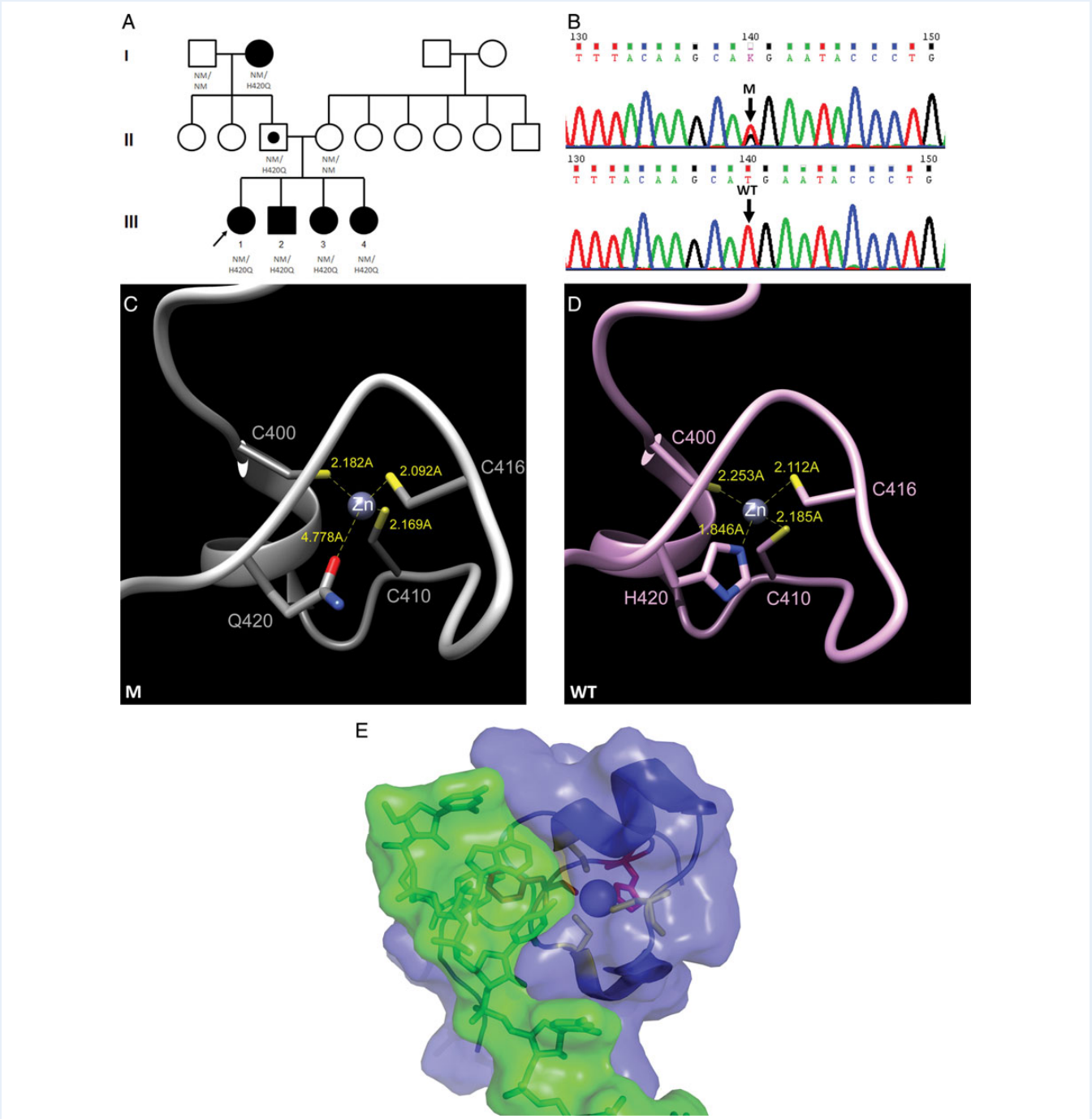

Figure 1 Genetic analysis of a family with central precocious puberty and an *in silico* model of the mutated protein. (**A**) Pedigree of the reported family. Black symbols—clinically affected family members, symbols with black point inside—asymptomatic carrier. The *MKRN3* genotype is shown for family members whose DNA was available for genetic studies. NM denotes non-mutated. (**B**) MKRN3 c.1260 T>G mutation analysis. Sequence chromatograph from genomic DNA of affected (M) and unaffected (WT) individuals. (**C** and **D**) An *in silico* structural model of the H420Q mutation. MKRN3 394–423 model structures of mutated (M) and wild-type (WT), presented as ribbons. C400, C410, C416 and H420 (Q in the mutant) are shown by sticks (Sulfur *yellow*, oxygen, *red*; nitrogen, *blue*). Zn ion is shown as Van der Waals sphere. Distances between Zn ion and cysteine's sulfur atoms, histidine's nitrogen atom and glutamine's oxygen atom were measured in angstroms and presented in the figure. Q420 is predicted to be more distant from the Zn ion (4.778 Å) than H420 (1.846 Å). (**E**) By way of example, the X-ray structure of a zinc finger from human butyrate response factor 2 in complex with AU-rich RNA molecule (PDB 1RGO) is shown. The protein is shown by blue ribbons and solvent accessible surface, with the three Zn-binding cysteines in yellow sticks and histidine in red sticks. Zn is shown by a gray sphere. A phenylalanine residue, close to the Zn binding site and pointing towards the RNA, is also shown by red sticks. This residue was found to be important for MKRN3 function.

CPP was diagnosed on the basis of clinical signs of progressive pubertal development before the age of 8 years in the girls; GnRH-stimulated LH levels, advanced bone age, and normal brain MRI.

Written informed consent was obtained from all family members according to a study protocol approved by the institutional ethical committee.

Genetic analysis

Whole exome sequencing

Exonic sequences were enriched in the DNA sample of patient III.4 using SureSelect Human All Exon 50Mb Kit (Agilent Technologies, Santa Clara, CA, USA). Sequences were determined by HiSeq2000 (Illumina, San Diego, CA, USA) as 100-bp paired-end runs. Data analysis including read alignment and variant calling was performed by DNAnexus software (Palo Alto, CA, USA) using the default parameters with the human genome assembly hg19 (GRCh37) as reference, as previously described (Edvardson *et al.*, 2012).

Sanger sequencing

We confirmed the identification of variants in the coding region of *MKRN3* with the use of PCR amplification followed by sequencing of the products using the conventional Sanger method (Genetic analyzer 3130, Applied Biosystems, Foster City, CA, USA). Genotypes of all other family members were determined by Sanger sequencing.

In silico *structural modeling*

Template structure for residues 394–423 of the MKRN3 protein was found using HHPRED: 4ii1 (chain A) and the wild-type (WT) structure was modeled using MODELLER (Sali and Blundell, 1993). 4ii1 is the protein data bank code for a solved structure for ZGPAT protein (zinc finger CCCH-type with G patch domain-containing protein).

The WT model was minimized in UCSF chimera (Pettersen *et al.*, 2004) using AMBER force-field, AM1-BCC charges while ZN ion, taken from 4ii1 PDB and structurally aligned to the MKRN3 model, was fixed in place.

Histidine 420 was mutated to glutamine using UCSF chimera swapaa command, and then minimized in the presence of a Zn ion, as for the WT model.

Results

Clinical characteristics

The proband (III.1) presented with thelarche at age 5.5 years, her sister (III.3) presented with thelarche and pubarche at age 5.5 years, and the youngest sister (III.4) presented with thelarche at age 4.5 years (Table I). Patient III.3 was followed by a pediatric endocrinologist for 11 months before referral to our center, during which period she progressed from breast Tanner 2 to 3. The parents brought in patient III.4 as soon as they noticed breast buds. The brother (III.2) presented at 9 years 10 months with advanced bone age, growth acceleration, testicular enlargement, penile length of 9.5 cm and Tanner stage 3 pubic hair. His first clinical presentation suggested the diagnosis of precocious puberty and his hormonal profile corroborated the diagnosis of CPP; puberty was estimated to have begun 2 years earlier.

All siblings had advanced bone age, accelerated growth velocity and pubertal levels of gonadal steroid and GnRH-stimulated LH.

Uterine width and volume were in the pubertal range (Herter *et al.*, 2002; de Vries *et al.*, 2006) in all three sisters.

All siblings were treated by GnRH analog(GnRHa) (3.75 mg of the long-acting GnRHa depot triptorelin) (Decapeptyl Depot, Ferring) and responded with regression of pubertal signs. The eldest sister discontinued

Table I Clinical and hormonal features of four siblings with central precocious puberty and makorin RING-finger protein 3 (MKRN3) mutation.

	Patient		III.1	III.2	III.3	III.4
	Sex		F	M	F	F
At onset	Age	Years	5.5	Unknown	5.5	4.5
	Growth velocity	cm/year	Unknown	11.4	10.5	9.9
At referral	Age	Years	5.8	9.8	6.3	4.5
	Bone age	Years	6.8	11.5	8.8	6
	Breast Tanner stage/testicular volume (ml)		3	12	3	2
	Pubarche stage		2	3	2	1
Hormonal profile	Basal LH	IU/l	2.4	1.3	0.4	0.2
	Peak LH		20.0	16.7	12.5	20.8
	Basal FSH	IU/l	8.9	2.61	5.5	3.9
	Peak FSH		17.1	3.25	13.4	16.9
	Estradiol	pmol/l	61		7.8	8.3
	Testosterone	nmol/l		6.9		
Brain magnetic resonance imaging			Normal	Normal	Normal	Normal
Pelvic ultrasound	Uterus	Length (cm)	4.1		3.2	3.2
		Width (cm)	2.5		1.9	1.8
		Volume (ml)	2.7		4.5	3.3
	Ovarian volume (ml)	Right	2.4		1	0.7
		Left	1.9		1.2	0.5

Normal prepubertal serum levels of testosterone and estradiol are <0.7 nmol/l and <3 pmol/l, respectively; the normal prepubertal basal serum level of LH is <0.1 IU/l, with a peak level <5.0 IU/l in both girls and boys. Levels were measured at the time of diagnosis. Normal pubertal levels of serum FSH have not been established because the normal ranges for pubertal and prepubertal FSH overlap. For central precocious puberty, cutoff values for uterine length range from 3.4 to 4.0 cm, uterine volume <2.0 ml, uterine width <1.5 cm and for ovarian volume range between 1 and 3 ml.

therapy at age 11 years, had her menarche at 12.5 years and currently has regular menses. The other three siblings are still being treated. The grandmother reported normal fertility and timely menopause.

Whole exome sequencing

The exome analysis of the DNA of patient III.4 yielded 65.00 million confidently mapped reads. Following alignment to the reference genome, 124 793 variants were noted. We removed variants which were called less than X8, were synonymous, present in the dbSNP (The Single Nucleotide Polymorphism Database) version 132, or in the Hadassah in-house database, or predicted benign by Mutation Taster software (Schwarz *et al.*, 2010). A total of 195 variants, all heterozygous, survived this filtering process; among them was chr15: 23812189 T>G, p.His420Gln (H420Q) in the *MKRN3* gene. The mutation was absent from dbSNP138 and from the 6503 healthy individuals whose Exome analysis results are available through the Exome Variant Server, NHLBI Exome Sequencing Project, Seattle, Washington, USA (http://evs.gs.washington.edu/ EVS-v.0.0.21) (accessed 28 January 2014). The mutation was verified by Sanger sequencing in all family members and was found to segregate with the disease in the family (Fig. 1A and B).

No rare or pathogenic variants were found in the KISS1R, KISS1, TAC3 and TACR3 (encoding tachykinin 3 and its receptor, respectively) genes. We reviewed all 195 potential single-nucleotide polymorphisms that were found, for genes that could be associated with the phenotype. MKRN3 was the only common gene found when comparing the list of variants with the list of genes known to be associated with puberty from the OMIM data base (http://www.ncbi.nlm.nih.gov/omim). In addition, an analysis of the list of genes with the DAVID bioinformatics tool (http://david.abcc.ncifcrf.gov/) failed to identify any gene supposedly involved in tumor suppression or puberty.

In silico *structural modeling of the mutation*

In the protein modeling the distances between zinc ion and cysteine's sulfur atoms, histidine's nitrogen atom and glutamine's oxygen atom were measured in angstroms (Fig. 1C and D). The Q420 mutated MKRN3 is predicted to be more distant from the Zn ion (4.778 Å) than the WT protein (1.846 Å).

To further analyze the possible effect of the mutation on the function of the MKRN3 protein, an X-ray structure of a similar zinc finger from another RNA binding protein, the human butyrate response factor 2, in complex with AU-rich RNA molecule (PDB 1RGO), was prepared (Fig. 1E). The Zn-binding site forms a pocket for the RNA, and the histidine is in close proximity with the zinc. Changing the histidine to glutamine is predicted to have a deleterious effect on the formation of this pocket, leading to dysfunction of the protein.

Discussion

This report describes a novel, heterozygous missense mutation in the maternally imprinted *MKRN3* gene in four affected siblings, their unaffected father and their affected paternal grandmother.

MKRN3 encodes makorin RING-finger protein 3, which is involved in ubiquitination and cell signaling. This gene is maternally imprinted and thus only the paternal allele is expressed (Jong *et al.*, 1999). The inheritance pattern in the studied family is in accordance with the expected mode of inheritance. The MKRN3 protein includes two copies of a C3H motif in the N-terminal, followed by a novel Cys–His configuration, a C3HC4 RING zinc finger, and a final C3H motif. Previously identified mutations in this gene consisted of seven frameshift mutations and four missense mutations: p.R365S (Abreu *et al.*, 2013), and p.C340G (Settas *et al.*, 2014) at the C3HC4 RING domain which are responsible for the ubiquitin ligase activity of the protein. Both missense mutations are predicted to disrupt protein function as an E3-ubiquitin ligase. Another missense mutation, F417I (Macedo *et al.*, 2014), was found in the last C3H1 domain, not far from the missense mutation described in the present study. The fourth mutation is a nonsense mutation at codon p.Glu111* at the first C3H motif (Schreiner *et al.*, 2014). As C3H zinc finger motifs have been implicated in RNA binding, we predict that the mutation found in the present study is responsible for disturbed RNA binding. To further elucidate the mechanism by which the mutation leads to a deleterious effect, we used an *in silico* structural model. We demonstrated that substituting histidine 420 with glutamine in the MKRN3 protein is predicted to cause a zinc-to-ligand distance longer than the longest distance estimated for bond lengths (Laitaoja *et al.*, 2013): the average length of the zinc-histidine bond is 2.09 Å and the longest distance found was 4.12 Å. Thus, the change is predicted to reduce the affinity between the Zn ion binding site and the relevant Zn, disrupting the binding pocket. In zinc finger proteins, the zinc ion is required for correct folding of the polypeptide chain, and removal of the zinc ion causes the finger to unfold (Krishna *et al.*, 2003).

In a study of the conserved CCCH zinc finger using a model of Euryarchaeotes (mesophilic archaeon *Methanosarcina acetivorans*), the N-terminal deletion mutant contained zinc at a level comparable to the WT protein level while the C-terminal deletion mutant was devoid of zinc (Lin *et al.*, 2005). The latter study supports our hypothesis regarding the mutation effect. This model brings us one step further towards understanding the as yet unknown mechanism by which MKRN3 is involved in the process of pubertal onset.

Puberty represents the reactivation of the suppressed GnRH pulse-generator characteristic of late infancy and childhood, leading to increased amplitude and frequency of GnRH pulsatile discharges. This change is related to an increase in stimulatory factors and decreased inhibitory tone, which are controlled by gene expression (Terasawa and Fernandez, 2001; Plant and Barker-Gibb, 2004).

It has been shown that the expression of *Mkrn3* in mice of both sexes was highest at post-natal day 10 and declined significantly just before the onset of puberty (Abreu *et al.*, 2013), suggesting that the MKRN3 protein may be involved in the restraint mechanism during childhood. We suggest that a low affinity to Zn leads to inadequate folding of the zinc finger, resulting in a reduced inhibitory effect of MKRN3 on GnRH secretion and earlier activation of puberty. The exact role of the MKRN3 protein and how it interacts with other inhibitory and excitatory neurotransmitters regulating puberty is unknown and definite confirmation that the missense variant causes premature loss of the inhibitory tonus acting on GnRH secretion awaits the availability of a functional assay for MKRN3. To date, there has been no direct evidence for a MKRN3/GnRH interaction or any biological testing for the consequences of the mutation on function of the MKRN3 protein. Moreover, there has been no documentation of the profiles of expression of MKRN3 in other reproductive tissues. Thus, the suggested role of MKRN3 remains only speculation and the possibility of additional actions at other levels of the reproductive axis cannot be ruled out.

In the present study, we used whole exome sequencing as a hypothesis-free approach to identify genetic variants. Among the 195

variants that survived the filtering process there was the intronless MKRN3 gene, recently found to be associated with familial CPP, and we focused on this gene in our study. We also reviewed the other 194 variants for mutations putatively affecting known pathways regulating puberty onset, such as LIN28B (Ong *et al.*, 2009).We did not find any gene that is known to be associated with precocious puberty, or with the pubertal process, apart from MKRN3.

It is not yet clear why clinical presentation occurs only at the age of 4 or 5 years and not earlier. The mutated MKRN3 protein may provoke an imbalance between stimulatory and inhibitory factors. It may be that the increase in stimulatory factors during childhood is gradual, and that when the inhibitory mechanism is impaired, as with MKRN3 mutation, the 'threshold' for puberty activation is lower. Conversely, it may be that the physiological decline in inhibitory factors is gradual, and that the switch occurs earlier in the absence of adequate MKRN3 activity. Clinically, it seems that the MKRN3 protein plays a role in determining pubertal timing, but not tempo, as observed in patient III.3, and possibly her brother (patient III.2). This is further supported by the normal course of pubertal progression observed in the eldest sibling (patient III.1, now 19 years old) following treatment cessation and the reported pubertal progression in the grandmother. Our observation is substantiated by the previous reports (Abreu *et al.*, 2013; Macedo *et al.*, 2014), showing that in some of the cases there was a significant lapse of time between initial clinical signs and the diagnosis of Tanner stage 2 or 3. For instance, in the manuscript of Abreu *et al.* (2013) a male patient whose pubertal signs appeared at the age of 5.9 years was at Tanner stage 3 at the age of 8.1 years, and two girls with two different mutations who had thelarche at the ages of 3.0 and 4.0 years were at Tanner 3 when diagnosed at the ages of 6.7 and 6.8, respectively (Macedo *et al.*, 2014). This observation may have clinical implications when considering GnRH agonist treatment for a child with precocious puberty associated with a MRKN3 mutation.

Although the timing of pubertal onset in the boy is uncertain, it seems that his degree of precocity was not as striking as in the girls. This gender dimorphism in the clinical presentation of the mutation in the MKRN3 gene is consistent with that found in recent studies (Abreu *et al.*, 2013; Settas *et al.*, 2014). The mechanism for such a gender difference is yet to be revealed.

In conclusion, a novel mutation, predicted to cause inadequate folding of the last zinc finger, is associated with familial precocious puberty. We suggest that MKRN3 signaling is a critical element in the restraint mechanism on the GnRH pulse-generator, maintaining the timing of the pubertal process.

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Authors' roles

L.d.V. developed the idea for the study, participated in the study design, collected the DNA samples, performed pelvic ultrasound, participated in interpretation of data and writing of the manuscript. G.G.-Y. performed analyses and participated in interpretation of data as well as writing of the manuscript. N.D. provided patients for the study and contributed to the analysis and interpretation of data and critical revision. A.S. contributed to the study design, interpretation of data and critical revision. M.P. contributed to the study design, interpretation of data and critical revision. All authors have approved the final version of the manuscript.

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Conflict of interest

None declared.

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