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Original Research

Familial central precocious puberty due to DLK1 deficiency: novel genetic findings and relevance of serum DLK1 levels

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Abstract

Background: Several rare loss-of-function mutations of delta-like noncanonical notch ligand 1 (DLK1) have been described in non-syndromic children with familial central precocious puberty (CPP).

Objective: We investigated genetic abnormalities of *DLK1* gene in a French cohort of children with idiopathic CPP. Additionally, we explored the pattern of DLK1 serum levels in patients with CPP and in healthy children at puberty, as well as in wild-type female mice.

Patients and Methods: Genomic DNA was obtained from 121 French index cases with CPP. Automated sequencing of the coding region of the *DLK1* gene was performed in all cases. Serum DLK1 levels were measured by enzyme linked immunosorbent assay (ELISA) in 209 individuals, including 191 with normal pubertal development and in female mice during postnatal pubertal maturation.

Results: We identified 2 rare pathogenic *DLK1* allelic variants: A stop gain variant (c.372C>A; p.Cys124X) and a start loss variant (c.2T>G; p.Met1?, or p.0) in 2 French girls with CPP. Mean serum DLK1 levels were similar between healthy children and idiopathic CPP children. In healthy individuals, DLK1 levels correlated with pubertal stage: In girls, DLK1 decreased between Tanner stages III and V, whereas in boys, DLK1 decreased between Tanner stages II and V ($P = .008$ and .016, respectively). Serum levels of Dlk1 also decreased in wild-type female mice.

Conclusions: Novel loss-of-function mutations in *DLK1* gene were identified in 2 French girls with CPP. Additionally, we demonstrated a pattern of dynamic changes in circulating DLK1 serum levels in humans and mice during pubertal stages, reinforcing the role of this factor in pubertal timing.

Keywords: central precocious puberty, DLK1 levels, *DLK1* mutations, pubertal timing

Significance

DLK1 has a crucial inhibitory role in the delta-notch pathway with impact in the reproductive and metabolic systems. *DLK1* deficiency is a rare monogenic cause of familial central precocious puberty (CPP) in children. Two distinct protein forms of DLK1 are recognized: Transmembrane and soluble forms. In this current study, we identified novel loss-of-function mutations in the maternally imprinted *DLK1* gene in 2 unrelated French girls with non-syndromic CPP. DLK1 circulating serum (soluble form) decreased during pubertal development in healthy girls and boys, as well as in wild-type female mice. These serum findings contrasted with the increasing of hypothalamic DLK1 expression at puberty in mice. DLK1 is a definitive inhibitor factor in the regulation of human pubertal timing.

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Introduction

The Delta-Notch pathway is an evolutionarily conserved signaling pathway, which participates in a broad range of developmental processes including cell proliferation, differentiation, and death.[1] Delta-like noncanonical notch ligand 1 (DLK1), also known as preadipocyte factor 1 or fetal antigen 1, is a membrane-bound protein with a crucial inhibitory role in the Delta-Notch pathway. The extracellular domain of DLK1 contains 6 tandem epidermal growth factor (EGF)-like repeats, followed by a juxta-membrane region with a TNF-α-converting enzyme, Disintegrin and Metalloproteinase Domain-Containing Protein 17—TACE (ADAM17), mediated cleavage site, a transmembrane domain, and a short intracellular tail.[2,3] When canonical ligands bind to Notch receptors, a sequence of conformational changes and enzymatic cleavages frees the Notch intracellular domain, which translocates to the nucleus and elicits a variety of cellular responses.[4] In contrast, when DLK1 binds to Notch receptors, it does not elicit the cleavage of the intracellular domain, inhibiting the signaling in a competitive manner.

In 2017, Dauber et al.[5] first identified a complex defect of the *DLK1* gene (~14-kb deletion and 269-bp duplication) in 5 members of a multigenerational Brazilian family with non-syndromic central precocious puberty (CPP) by linkage analysis and whole-genome sequencing strategies. Subsequently, novel frameshift pathogenic allelic variants located in the extracellular domain of DLK1 were identified in several women with CPP/precocious menarche from Brazil, United Kingdom, and Spain.[6,7] Metabolic abnormalities in adulthood phase, such as overweight/obesity, early-onset glucose intolerance/type 2 diabetes mellitus, and hyperlipidemia, were more prevalent in the women with *DLK1* pathogenic allelic variants than in woman from an idiopathic CPP group.[6] The high prevalence of metabolic alterations in adult women who experienced precocious puberty due to *DLK1* defects suggested that DLK1 represents a link between reproduction and metabolism.[6]

A soluble form of DLK1 with a molecular weight of 50 kDa can be generated through TACE (ADAM17)-mediated cleavage of its extracellular domain, making DLK1 a measurable serum protein. Serum DLK1 concentrations were undetectable in girls and women with CPP caused by loss-of-function mutations of *DLK1*, suggesting that this accessible biochemical measurement could be a potential screening assay for the diagnosis of CPP due to a rare deficiency of this protein.[5–7] Interestingly, a study in Danish men with a median age of 68 years showed that serum DLK1 levels correlated positively with body mass index (BMI), total fat mass, fat mass percentage, homeostasis model assessment of insulin resistance (HOMA-IR), and adipose tissue insulin resistance index (Adipo-IR), while they correlated negatively with insulin sensitivity.[8] Of note, *Dlk1* deficient mice achieved puberty at a considerably lower body weight, suggesting that the lack of *Dlk1* could attenuate the effect of the low body weight on determining pubertal onset in these animal models.[9] These findings enhance the possible correlation between *DLK1* biology and metabolic features.

In the current study, we investigated potential genetic defects and serum levels of DLK1 in children with idiopathic CPP. We also analyzed serum DLK1 levels in healthy children in different pubertal stages and compared them to those children with CPP. Furthermore, we analyzed serum Dlk1 levels in peripubertal female mice.

Patients

Genomic DNA was obtained from 121 French individuals with CPP (98 girls and 23 boys). This European cohort was evaluated and organized by 1 coauthor (R.B.) from Université Paris Cité, Paris, France. This study was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all patients and their legal guardians.

All patients had precocious puberty, defined by the development of progressive pubertal features according to Marshall and Tanner pubertal staging before 8 years in girls or before 9 years in boys. The diagnosis of CPP was confirmed in all of them by measuring the LH peak after a GnRH stimulation test. All patients had advanced bone age (BA) (Greulich and Pyle method) compared to chronological age. In all cases, brain magnetic resonance imaging (MRI) showed no lesion that could lead to organic CPP.

For DLK1 serum concentration analysis, blood samples were collected from 209 individuals (115 females and 94 males; 20 Brazilian and 189 Spanish) at different pubertal stages. Among these individuals, 18 were girls with CPP and the remaining 191 had normal pubertal development.

DNA sequencing

DNA was collected from index cases, their parents, and first- or second-degree family members when available. Genomic DNA was extracted from peripheral blood lymphocytes according to standard protocols. The *DLK1* gene (5 exons—GenBank accession number NM_003836) was amplified by polymerase chain reaction (PCR) followed by purification and automated sequencing of the products using the Sanger sequencing method. DNA sequences obtained were compared to the human GenBank *DLK1* sequence using Sequencher (Gene Codes Corporation, Ann Arbor, MI) sequence alignment software. The PCR primers and conditions are available upon request.

Allelic variants were classified as pathogenic, likely pathogenic, variant of uncertain significance, likely benign or benign according to criteria established by the American College of Medical Genetics and Genomics (ACMG).[10]

In silico analysis

The ACMG classification and the pathogenicity prediction site analysis were performed using Varsome[11] and Franklin by Genoox (Palo Alto, CA). The Genome Aggregation Database (gnomAD; https://gnomad.broadinstitute.org)[12] and the Brazilian Genomic Variants database (ABraOM; https://abraom.ib.usp.br) were included for allele frequency analysis.[13] Candidate variants were evaluated for submissions in the international literature and in ClinVar, a public archive of human variations and related phenotypes (https://www.ncbi.nlm.nih.gov/clinvar/).

Serum DLK1 measurements in humans

Enzyme linked immunosorbent assay (ELISA; IB99504, IBL-America, MN, USA) was used for DLK1 serum analysis. All steps were performed following the manufacturer's instructions. The sensitivity of the assay was set at 0.336 ng/mL. Inter-assay coefficient of variability (CV) was 4.6%, and intra-assay variability was 5.1% according to the

manufacturer information. Data were analyzed according to pubertal staging, BMI, age, and sex.

Serum DLK1 measurements in mice

Five wild-type female C57BL/6 mice were monitored for pubertal signs including vaginal opening (VO—pubertal onset) and first estrus (FE—pubertal maturity). Serum was obtained from whole blood collected at: (1) Weaning (21 days of life), (2) VO, (3) 5 days after VO, (4) FE, and (5) 5 days after FE. Serum Dlk1 was measured by ELISA (EM66RB, Invitrogen, USA). All steps were performed following the manufacturer's instructions. The sensitivity of the assay was 0.0045 ng/mL. Inter-assay CV was <12%, and intra-assay variability was <10%, according to the manufacturer's information.

Statistical analysis

Descriptive statistics or frequencies and percentages were calculated for all numerical or categorical variables. Data were presented as mean and standard deviation (SD) unless otherwise stated. For human data, comparisons were made through Student's *t*-test or Wilcoxon signed-rank test for numerical continuous variables as appropriate. Categorical variables were compared between groups through Chi-square test or Fisher's exact test as appropriate. Linear regression analysis was performed to evaluate relationship between a scalar response and 1 or more explanatory variables. Statistical analysis was performed in R Studio (version 1.2.1335). Regarding the mouse data, statistical analysis of the results obtained at the different pubertal stages was performed using 1-way analysis of variance (ANOVA), and data were analyzed using Prism statistics software (GraphPad, Inc., San Diego, CA). A *P*-value less than .05 was considered statistically significant for both human and mouse data.

Results

DNA sequencing

We identified a rare stop gain allelic variant (c.372C>A; p. Cys124X; rs749564412) in a French girl (patient 1) with non-syndromic CPP. This variant was inherited from her asymptomatic father (Figure 1). This nucleotide change was located in exon 4 (Figure 2), which encodes the third EGF-like repeat in the extracellular domain of the protein, leading to a change of a cysteine to a premature stop codon. This rare allelic variant had a minor allelic frequency (MAF) of 0.000003983 in gnomAD and was absent in ABraOM and in ClinVar. Therefore, it was classified as pathogenic according to the ACMG criteria. This female patient was the only child of non-consanguineous parents. She was born at term (40 weeks) with a birth weight of 2830 g (−1.4 SDS) and birth length 45.5 cm (−3.0 SDS). Hence, she was small for gestational age (SGA)

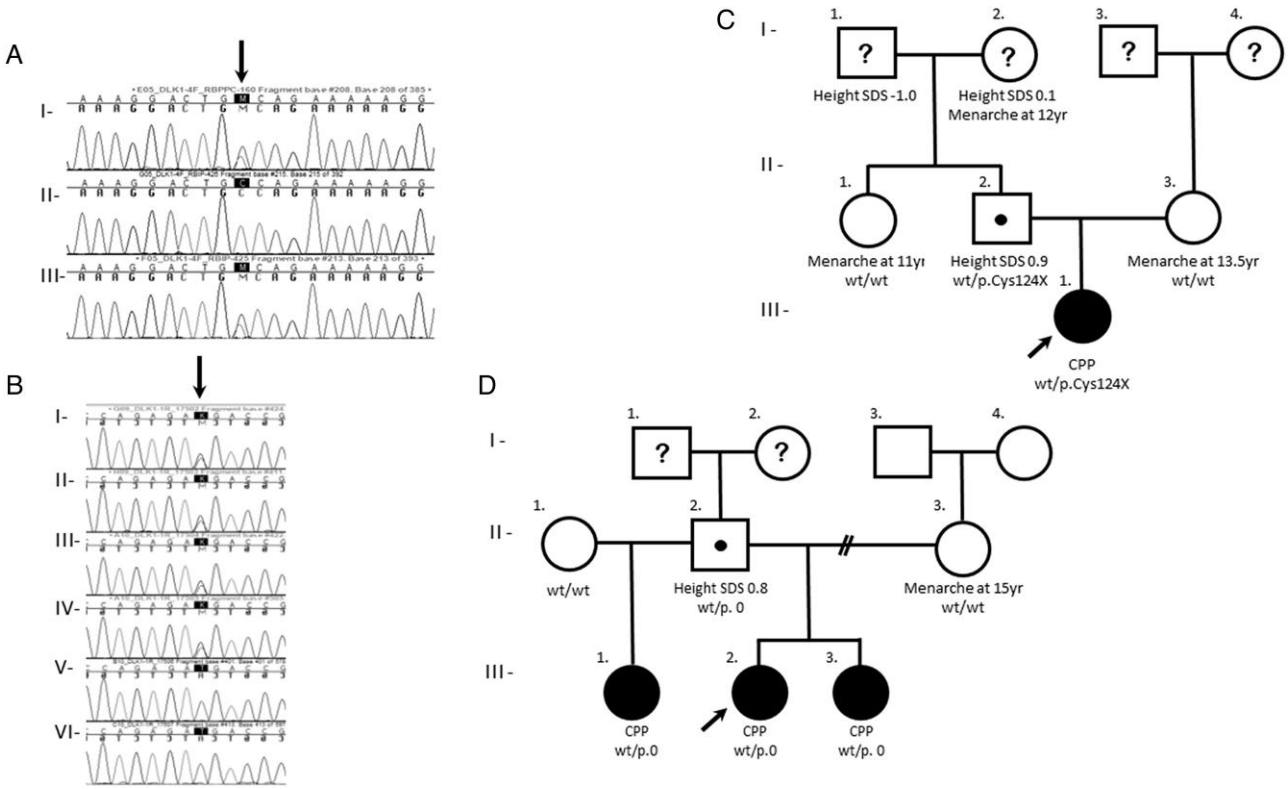

Figure 1. Pedigrees of the 2 families and sequencing data of the *DLK1* gene showing rare allelic variants. A. Electropherogram of index patient 1 and her relatives. I) Index case harboring the *DLK1* pathogenic allelic variant c.372C>A; p.Cys124X—(rs749564412). II) Normal electropherogram of her mother. III) Her father is an asymptomatic carrier of the mutation. B. Electropherogram of index patient 2 and her relatives. I) Index case harboring the *DLK1* pathogenic allelic variant c.2T>G p.Met1?, or p.0. II) The father of index case 2, harboring the pathogenic allelic variant and transmitting it to the index case, to the sister, and to her half-sister. III and IV) Paternal half-sisters of the index patient 2. V and VI) Normal electropherogram of the index's mother and the paternal half-sister's mother, respectively. C and D. Family pedigrees of patients 1 and 2, respectively. Squares indicate male members, circles female members, solid symbols affected members, dot symbol indicates that the subject is a carrier, and question marks indicate members who were not evaluated. The index case is indicated by an arrow. CPP, central precocious puberty.

for length (Usher and McLean method). She had appropriate neuro-psychomotor development. Accelerated growth was noticed at 3 years, followed by thelarche at 4.5 years. She was first evaluated at 5.5 years old when she was Tanner IV for breast development, had a height of 114.5 cm (0.85 SDS), and BMI in the obesity range (2.48 SDS). At this time, her basal and GnRH-stimulated LH levels were both in the pubertal range. Brain MRI had no anatomical abnormality. Serum was not available for DLK1 serum assessment. Her parents had normal height (father height SDS 0.9 and mother height SDS 0.9), and no history of premature sexual development.

Additionally, we identified a rare start loss variant (DLK1: c.2T>G; p.Met1?; or p.0) in a French girl (patient 2) with familial CPP (Figure 1). This nucleotide change was located in exon 1 that encodes the signal peptide, leading to loss of the start codon (AUG) (Figure 2). Familial segregation analysis showed that this allelic variant was inherited from her asymptomatic father, and it was present in her affected sister and paternal half-sister (Figure 1). This rare allelic variant was absent in gnomAD and ABraOM, as well as in ClinVar. The variant was classified as likely pathogenic according to the ACMG criteria. This female patient was born at term with appropriate weight (3200 g; −0.4 SDS) and length (49 cm; −0.9 SDS). At 6 years old, precocious thelarche was first noticed and she had a BA of 7 years; her height was 120 cm (1.3 SDS), and her BMI was 14.2 kg/m^2 (−0.9 SDS). At this time, her basal and GnRH-stimulated LH levels were in the pubertal range. Brain MRI had no anatomical abnormality. At adult phase (25 years old), her serum DLK1 levels were very low (0.37 ng/mL). Family history revealed that her sister had early menarche (10.5 years). Additionally, her paternal half-sister had a history of premature sexual development (thelarche at 6 years) and medical investigation confirmed CPP, characterizing a familial form of CPP.

Serum DLK1 levels in humans

Among the 209 individuals (115 girls, 94 boys) from the Brazilian and Spanish cohorts, 18 had idiopathic CPP and 191 had normal pubertal development. The cohort mean age was 14.7 ± 6.5 years. Mean serum DLK1 levels did not differ between idiopathic CPP and normal puberty (7.9 ± 3.6 and 8.2 ± 3 ng/mL, $P = .79$, Figure 3). In healthy children ($n = 191$), a distinct pattern according to the Tanner staging was observed. In girls ($n = 97$), DLK1 levels decreased from Tanner III (TIII) to Tanner V (TV) (Figure 4A, $P = .016$ for TIII vs TV). In boys ($n = 94$), DLK1 levels decreased from TII to TV ($P = .008$ for TII vs TV) (Figure 4B).

Linear regression analysis of serum DLK1 levels and BMI SDS showed no association in either girls (Figure 5A; $P = .54$) or boys (Figure 5B; $P = .33$) in this pediatric cohort. Additionally, categorical stratification of serum DLK1 levels by obese vs normal weight did not statistically differ (Figure 5C).

Serum Dlk1 levels in mice

Five wild-type female C57BL/6 mice were evaluated and achieved VO at age 25.6 ± 1.9 days and FE at age 37.6 ± 2.6 days (mean ± SEM). Serum Dlk1 levels were highest at weaning (age 21 days, 1.9 ± 0.2 ng/mL [mean ± SEM]), decreasing progressively throughout pubertal maturation, achieving the lowest levels 5 days after FE (0.1 ± 0.02 ng/mL) (Figure 6). Serum Dlk1 levels were statistically different among the 5 different pubertal stages evaluated, except when comparing the levels at FE to those 5 days after FE ($P = .99$).

Discussion

In the current study, we described 2 novel loss-of-function mutations in *DLK1* that caused a CPP phenotype in 2 girls from a

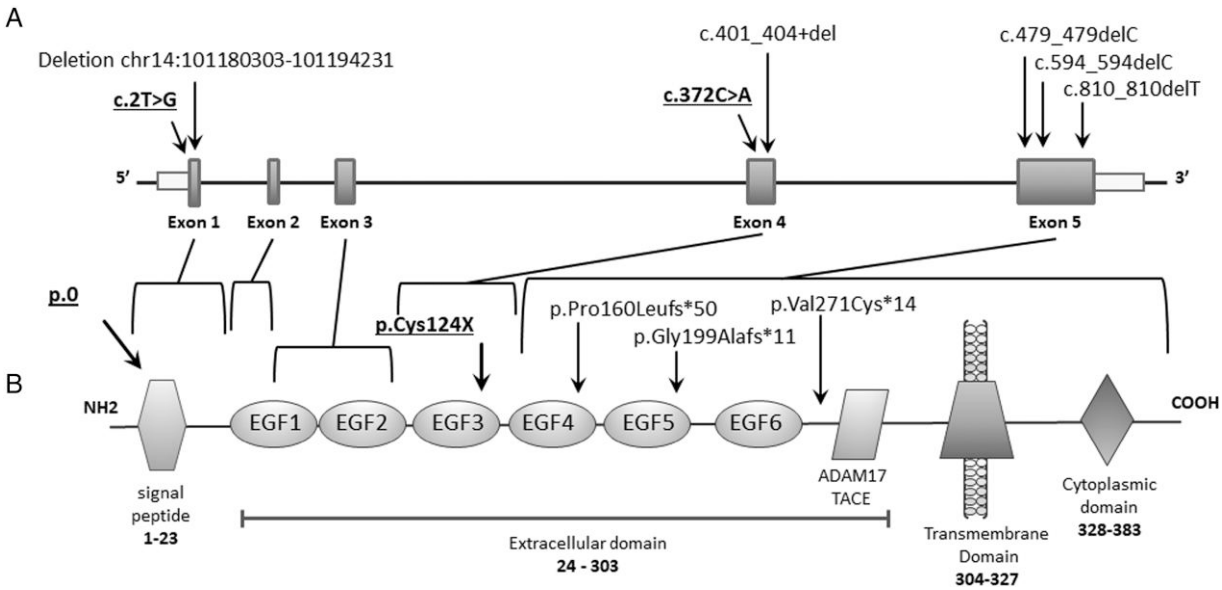

Figure 2. Schematic representation of the human *DLK1* gene and human DLK1 protein. A. Human *DLK1* gene (transcript ENST00000341267.9): The boxes indicate the coding sequences of the 5 exons of the gene in humans. Open boxes indicate the 5′- and 3′-untranslated regions of the gene, respectively. Previously described mutations are indicated by arrows. The 2 new mutations found in this work are indicated by arrows in bold. B. Human DLK1 protein structure (P80370): The hexagon indicates the signaling peptide; circles the 6 EGF-like repeats. The rhomboid indicates the extracellular TACE (ADAM17) proteolytic cleavage domain. The trapeze figure indicates the transmembrane domain, and the diamond represents the cytoplasmic domain. The numbers below the figure represent the amino acid positions of the indicated domains. Corresponding protein sequences of the previously mutations described are shown by arrows. The 2 new mutations found in this work are indicated by arrows in bold. EGF, epidermal growth factor (EGF).

large French cohort. Both female patients had non-syndromic CPP with premature and progressive breast development associated with accelerated growth, advanced bone age, and pubertal basal and GnRH-stimulated LH levels. Patient 1 was born SGA for length, while patient 2 was appropriate for gestational age. It is worth noting that murine models of Dlk1 deficiency had consistently low birth weight and/or length.[14] However, in humans, this feature had not been identified, at least so far, as most patients with loss-of-function mutations in *DLK1* were born appropriate for gestational age.[6,15] In addition, *DLK1* mutations have not been identified in short stature-SGA cohorts.[16]

Both patients inherited the *DLK1* mutations from their unaffected carrier fathers, following an autosomal dominant inheritance with imprinted pattern with paternal transmission. Patient 1 had a CPP form that firstly appeared to be sporadic; however, the genetic analysis uncovered a paternally inherited *DLK1* mutation. Meanwhile, patient 2 had a familial form of CPP with 2 affected sisters and familial segregation analysis confirmed that all affected siblings carried a *DLK1* paternally inherited mutation. Our previous and current findings showed that the clinical features of CPP caused by DLK1 deficiency are relatively indistinct from other causes of CPP.[5–7] These evidences reinforce the importance of the genetic evaluation of patients with idiopathic CPP. Very low DLK1 serum levels, however, could represent a potential screening tool.

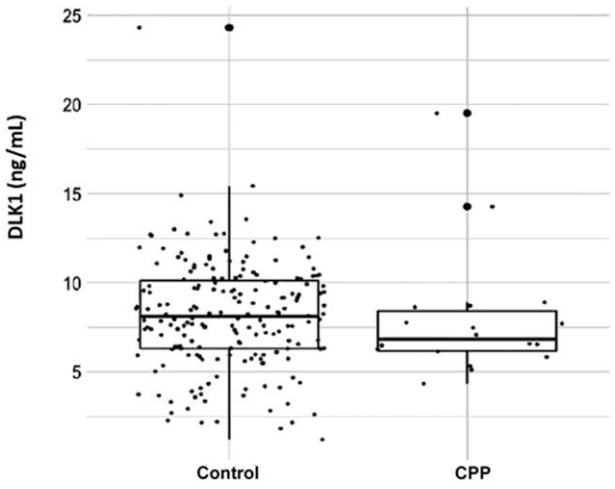

Figure 3. Serum DLK1 levels in normal puberty vs idiopathic CPP. Box plots represent the serum DLK1 levels in normal puberty subjects (control) and in CPP subjects. CPP, central precocious puberty.

Patient 1 had CPP associated with obesity. An increased prevalence of obesity in DLK1 deficient patients can occur in adulthood, rather than early childhood, and it is probably influenced by several other environmental, behavioral, social, and genetic characteristics, as is the case with a complex disease such as obesity.[6] As DLK1 prevents adipocyte maturation, its loss presumably leads to a facilitated expansion of fat mass, as suggested in several animal models of Dlk1 deficiency.[15,17]

The large majority of cases of CPP associated with *DLK1* mutations to date have been in females, although there is no known genetic mechanism that could prevent boys from developing the phenotype. It is well documented that idiopathic CPP is more commonly diagnosed and more prevalent in girls, which could suggest this bias as a determinant factor in this observation.[18] Recently, a Chinese boy with familial CPP, overweight, hyperlipidemia, and hyperuricemia was shown to harbor a paternally inherited frameshift mutation in *DLK1* (NM_003836.5: c.479delC—p.Pro160Leufs*50), previously described in a Brazilian family.[6,19] In addition, a novel heterozygous frameshift mutation in *DLK1*, c.288_289insC; p.Cys97Leufs*16, was identified in a male proband in an Italian study.[20] Familial segregation analysis showed that the variant was inherited from his affected and untreated father, and that it was also present in his affected sister. At adulthood, the untreated father had short stature, as well as hypercholesterolemia, an unfavorable metabolic outcome related to DLK1 deficiency.

While serum DLK1 levels in the current study, as well as in the Italian study did not correlate with BMI SDS, other studies

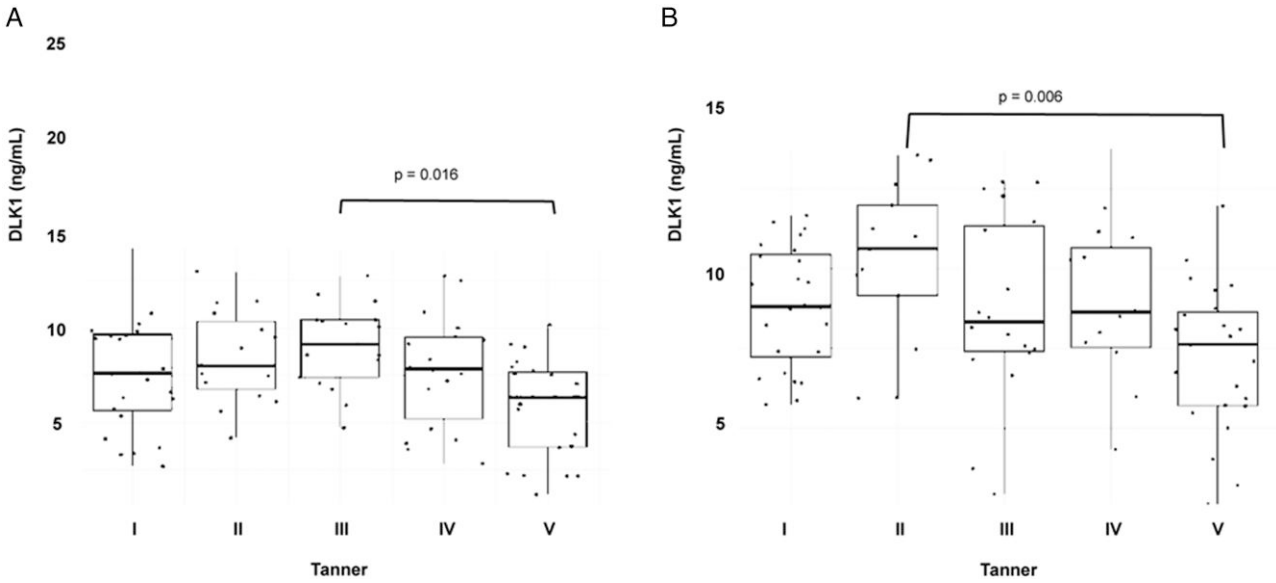

Figure 4. DLK1 levels in girls and boys according to Tanner stages. DLK1 levels according to puberty Tanner stages in (A) girls and (B) boys.

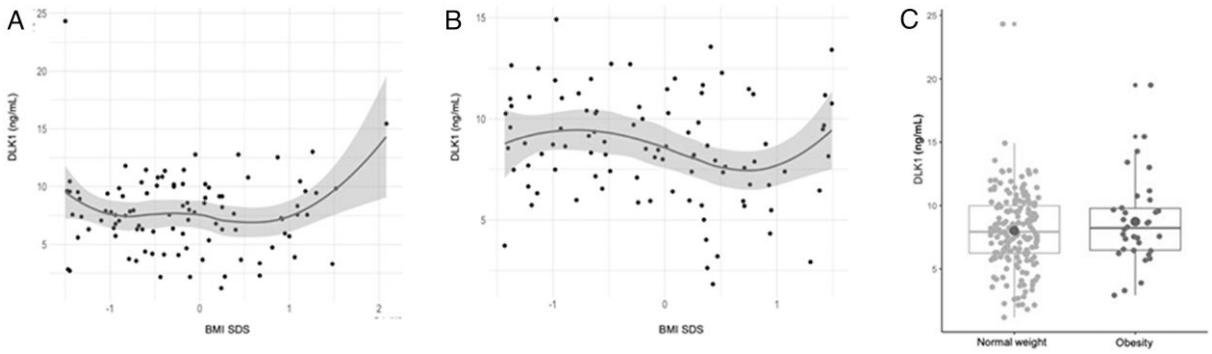

Figure 5. DLK1 according to BMI SDS and age. A. DLK1 levels vs BMI SDS in girls. B. DLK1 levels vs BMI SDS in boys. C. DLK1 levels in obese/overweight vs normal weight subjects. BMI, body mass index.

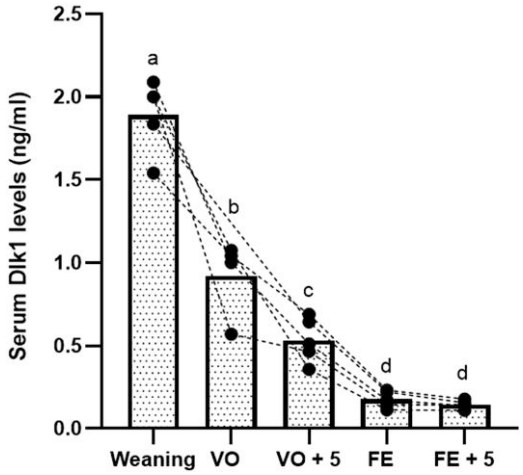

Figure 6. Circulating serum Dlk1 levels in peripubertal female mice. Serum Dlk1 levels of 5 female mice followed longitudinally across pubertal maturation. The mice were all weaned at age 21 days. Vaginal opening (VO) occurred at age 25.6 ± 1.9 days (mean ± SEM), and first estrous (FE) at age 37.6 ± 2.6 days (mean ± SEM). The dots connected by lines represent the same animal at the different pubertal stages, and the bars indicate the mean level at each stage (considering all mice together). Significant differences between serum Dlk1 levels at the various pubertal stages assessed are indicated with different letters (a to d) ($P < .05$), as determined by 1-way ANOVA.

have previously described a positive correlation between these factors.[6,21,22] These previous reports evaluated older patients, with a mean age ranging from 32 to 68 years. Meanwhile, in our cohort, the mean age was 14.7 ± 6.5 years, which could explain why this positive correlation was not observed, as it might become more evident in later stages of life. Additionally, the comparison between patients with CPP vs normal puberty showed no difference regarding the DLK1 levels, however, the number of patients in the CPP group was lower and had a higher spread with some outliers. Complete DLK1 deficiency resulted in a higher prevalence of obesity and metabolic syndrome. Interestingly, in patients with normal DLK1 synthesis, there was a positive correlation between DLK1 levels and BMI. This pattern was observed also in adipokines, such as leptin. Leptin deficiency is a known cause of genetic obesity, yet in normal subjects, leptin levels increase with BMI and can result in leptin resistance in obese subjects.[23] As DLK1 is an inhibitor of adipocyte maturation, it is possible to hypothesize that, while in deficient DLK1 individuals, there is a more permissive environment to fat mass expansion, in healthy individuals, DLK1 levels increase with fat mass expansion, resembling a negative feedback mechanism.[8] However, this mechanism is presumably ineffective in individuals with high BMI.

To our knowledge, our study is the first to describe serum DLK1 levels throughout human and mouse pubertal stages. Villanueva *et al.*[24] showed an increase in *Dlk1* and *Kiss1* expression between prepubertal and pubertal/adulthood developmental phases in the mouse hypothalamus. Furthermore, this study demonstrated that Dlk1 was expressed almost exclusively as a soluble protein. In the current study, we demonstrate that circulating serum Dlk1 levels decreased across mice and human pubertal maturation, in an opposite pattern to what was previously reported in the hypothalamus expression.[24] This decrease in serum DLK1 levels seen during pubertal development was statistically significant in girls between Tanner stages III and V and in boys between Tanner stages II and V, although there were overlapping values between Tanner stages I and II in both sexes. Notably, DLK1 is expressed in several tissues, especially in endocrine glands (adrenal, pancreas, ovaries, and pituitary) and adipose tissue. We believe that the circulating DLK1 levels are not probably originated from central nervous system (hypothalamus), and the adipocyte tissue could be a main source during pubertal development. Previously, a French study measured the circulating levels of DLK1 in 38 healthy children aged 0-17 years. They found that serum DLK1 levels decreased progressively from birth to late adolescence. In our current study, we evaluated specifically the pubertal age range and focused on the distinction between DLK1 levels throughout pubertal maturation.[25] These preliminary findings suggested that peripheral circulating DLK1 might have a role in pubertal onset regulation.

Altogether, this study demonstrated 2 novel *DLK1* mutations in French patients to be added to the growing multiethnic cohort of CPP caused by loss-of-function mutations in *DLK1*. Furthermore, we demonstrated a distinct pattern of circulating serum DLK1 levels throughout puberty in healthy individuals and in female mice. The dynamic relationship between these *DLK1* levels and the pubertal stages further corroborates its role in pubertal development.

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