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CARLOS EDUARDO SERAPHIM

Investigação dos aspectos metabólicos da puberdade precoce central

SÃO PAULO

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Investigação dos aspectos metabólicos da puberdade precoce central

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Programa de Endocrinologia

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DEDICATÓRIA

À minha **mãe**,
meu alicerce emocional e
fonte de força inesgotável.
Ao meu **pai,**
que muito se entregou
e muito cedo partiu.
A meus irmãos,
meus primeiros e maiores amigos.

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EPÍGRAFE

"Ninguém ignora tudo. Ninguém sabe tudo.

Todos nós sabemos alguma coisa.

Todos nós ignoramos alguma coisa.

Por isso aprendemos sempre"

Paulo Freire

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Abreviatura dos títulos dos periódicos de acordo com *List of Journals Indexed in Index Medicus.*

SUMÁRIO

LISTA DE SIGLAS E ABREVIATURAS

a.a.	Aminoácido
ADAM	Proteína Desintegrina e Metaloproteínase
ADIPO-IR	Índice de resistência à insulina no tecido adiposo
aGnRH	Análogo do hormônio hipotalâmico liberador de gonadotrofinas.
AMPK	Proteína quinase ativada por AMP
ARC	Núcleo arqueado do hipotálamo
AVPV	Área periventricular anteroventral do hipotálamo
CAC	Escore de cálcio coronariano
CAPPesq	Comissão de Ética para Análise em Pesquisa
cDNA	DNA complementar
cm	Centímetros
DIO3	Dediodinase tipo 3 de iodotironina
DLK1	Gene que codifica a proteína homóloga *Delta-like1*
DLK1-DMR	Região diferencialmente metilada do *DLK1*
DM2	Diabetes mellitus tipo2
DMR	Regiões diferencialmente metiladas (*differentially methylated regions*)
DNA	Ácido desoxirribonucléico
DP	Desvio-padrão
DSL	Domínio tipo Delta, Serrate, e Lag2
Dyn	Dinorfina
E2	Estradiol
E3 ligase	Ligases de ubiquitina tipo E3
ECLIA	Ensaio eletroquimioluminescência
EED	Cofator repressor membro da família polycomb
EGF-like	Fator de crescimento semelhante ao da epiderme
F	Feminino
FA-1	Antígeno fetal tipo 1
FMUSP	Faculdade de medicina da USP

FOXO1	Proteína O1 da caixa forkhead
FSH	Hormônio folículo-estimulante
FTO	Gene que codifica a proteína da obesidade e de massa de gordura associada ou alpha-ketoglutarate-dependente dioxigenase
G	Medida de força centrífuga gravitacional
GABA	Ácido-γ-aminobutírico
GLP-1	Peptídeo semelhante a glucagon 1
GLT2/MEG3	Gene trap locus 2/maternally expressed gene 3
GnRH	Hormônio hipotalâmico liberador de gonadotrofinas
GWAS	Estudos de associação de polimorfismo envolvendo o genoma completo
HAS	Hipertensão arterial sistêmica
HCFMUSP	Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo
HDL	Lipoproteína de alta densidade
HOMA-IR	"Homeostatic Model Assessment for Insulin Resistance", parâmetro de resistência à insulina
IC	Idade cronológica
IFMA	Ensaio imunofluorométrico
IG-DMR	Região intergênica diferencialmente metilada
IGF-1	Fator de crescimento semelhante à insulina tipo 1
IMC	Índice de massa corporal
IO	Idade óssea
kb	Quilobases
kg	Quilogramas
KISS1	Gene que codifica a kisspeptina
KISS1R	Gene que codifica o receptor da kisspeptina
KNDy	Neurônio hipotalâmico produtor de kisspeptina, neurocinina e dinorfina
L	Litro
LC – MS/MS	Cromatografia líquida acoplada a espectrometria de massa
LDL	Lipoproteína de baixa densidade
LH	Hormônio luteinizante

LIN28B	Gene que codifica a proteína homóloga lin-28 tipo b
MAPK	Proteína quinase ativada por mitógenos
mg	Miligramas
MIRG	Gene contendo miRNA
miRNA	Micro RNA
MKRN3	Gene que codifica a proteína *makorin ring finger 3*
mRNA	RNA mensageiro
mTOR	Alvo da rapamicina em mamíferos
NKB	Neurocinina B
NOTCH1	Receptor 1 da família notch
NPY	Neuropeptídio Y
NPYR	Gene que codifica o receptor do neuropeptídio Y
NRR	Região regulatória negativa
ob/ob	Camundongo com mutação inativadora bialélica no gene responsável pela produção de leptina
p	Valor-p ou probabilidade de significância
pb	Pares de base
PCOS	Polycistic ovary syndrome (síndrome dos ovários policísticos)
PCR	Reação em cadeia da polimerase
POL2	RNA polimerase 2
PP	Puberdade precoce
PPC	Puberdade precoce central
PPP	Puberdade precoce periférica
PREF-1	Fator pré adipócito 1
RM	Ressonância magnética
RNA	Ácido ribonucleico
RT-qPCR	Reação em cadeia da polimerase em tempo real
RTL-1	Retrotransposon-like 1
SAT	Gordura abdominal subcutânea
SHBG	Globulina ligadora de esteroides sexuais
SIRT1	Sirtuína
SNC	Sistema nervoso central
snoRNA	RNA pequeno e nucleolar

TAC3	Gene que codifica a preprotaquicinina 3
TACE	Enzima conversora de fator de necrose tumoral α, também conhecida como desintegrina e metaloproteinase 17 (ADAM17)
TC	Tomografia computadorizada
UA	Unidades Agatston
UH	Unidades Hounsfield
UI	Unidade internacional
UTR	Região não traduzida
VAT	Gordura visceral abdominal
Z	Escore-Z
µg	Micrograma

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RESUMO

Seraphim CE. Investigação dos aspectos metabólicos da puberdade precoce central [tese]. São Paulo: Faculdade de Medicina, Universidade de São Paulo; 2023.

Contexto: A puberdade precoce é caracterizada pelo surgimento dos caracteres sexuais secundários de forma progressiva antes dos oito anos em meninas e nove anos em meninos, e pode ser central ou periférica. A puberdade precoce central (PPC) se divide em orgânica, idiopática ou genética. Dentre as causas genéticas, encontram-se as mutações inativadoras do gene *DLK1*, que codifica uma proteína transmembrana que age como inibidor competitivo dos receptores NOTCH. Indivíduos com mutações do *DLK1* apresentaram PPC e fenótipo metabólico anormal na fase adulta, com obesidade, diabetes mellitus tipo 2 precoce e síndrome dos ovários policísticos. Além disso, estudos em animais associam a perda do gene a maior quantidade de tecido adiposo, resistência à insulina e esteatose hepática. **Objetivo:** Descrever as características metabólicas dos pacientes com PPC, em especial dos casos associados as mutações inativadoras do *DLK1*. Avaliar a correlação da dosagem do DLK1 sérico com diferentes períodos puberais e estudar os impactos das mutações no *DLK1* no metabolismo de células in vivo de pacientes afetadas. **Métodos:** Análise clínica retrospectiva e prospectiva de uma grande coorte de pacientes com PPC, incluindo os primeiros casos descritos de mutações do *DLK1*. Comparação dos fenótipos clínicos/metabólicos entre as distintas causas de PPC, e da dosagem de DLK1 sérico em cada etiologia. Além disso, realizamos a análise do comportamento do DLK1 sérico em diferentes fases da puberdade. Em pacientes com mutações do *DLK1*, análise metabólica de células *in vivo*, de função mitocondrial, perfil de polarização imune e oxigenação celular, em comparação com controles saudáveis. **Resultados:** São apresentados 5 artigos, sendo os 4 primeiros publicados anteriormente e o último em processo de submissão. Cada artigo aborda um diferente aspecto analisado na presente tese. **Conclusões:** O fenótipo metabólico de pacientes com mutações do MKRN3 é indistinto de outras causas idiopáticas. A dosagem sérica do DLK1 evidencia um padrão dinâmico, com queda ao longo da progressão da puberdade. A deficiência de DLK1 leva a um metabolismo celular prejudicado, com maior formação de superóxido, perfil de polarização de macrófagos mais inflamatório, e prejuízo da respiração celular.

Palavras-chave: Puberdade precoce. Mutação genética. Metabolismo. Proteína 2 de ligação a metil-CpG**.** Síndrome metabólica.

ABSTRACT

Seraphim CE. Analysis of the clinical and metabolic aspects of central precocious puberty [thesis]. São Paulo: "Faculdade de Medicina, Universidade de São Paulo"; 2023.

Context: Precocious puberty is characterized by the progressive development of secondary sexual characteristics, before 8 yrs in girls and 9 yrs in boys and can be classified as central or peripheric. Central precocious puberty (CPP) is subclassified as organic, idiopathic, or genetic. One of the genetic causes is due to loss-of-function mutations in *DLK1*, a gene that translates into a transmembrane protein, which acts as a competitive inhibitor of NOTCH signaling. Subjects with these mutations presented CPP associated with a metabolic phenotype in adult life, with obesity, precocious type 2 diabetes and polycystic ovary syndrome. Furthermore, several animal studies have associated the loss of this gene with increased adiposity, insulin resistance and liver steatosis. The description of *DLK1* mutations created a new interface in the complex relation between pubertal axis and metabolism. **Objective:** To describe the metabolic features of subjects with CPP, especially those caused by loss-of-function mutations in *DLK1*. To evaluate the correlation of serum DLK1 levels with different puberty stages and to study the cellular metabolic impacts of the mutations of *DLK1* in affected subjects. **Methods:** Retrospective and prospective clinical analysis in a large cohort of subjects with CPP, including the first described cases of those caused by *DLK1* mutations. Comparison between the phenotypes of distinct causes of CPP, and the DLK1 serum levels in each cause. Furthermore, DLK1 levels were evaluated in each pubertal stage. In patients with *DLK1* mutations, metabolic in vivo cell analysis was made, including mitochondrial function, immune polarization profile, and cellular oxygenation, all compared to healthy controls. **Results:** Five manuscripts are presented, with the first four being already published and the last one in submission. Each manuscript describes a different aspect in the current thesis. **Conclusions:** The metabolic phenotype of patients with MKRN3 mutations is indistinct from other idiopathic causes. Serum DLK1 levels presents a dynamic behavior, with a decrease pattern as the progression of different pubertal stages occurs. DLK1 deficiency leads to impairment of cellular metabolism, with increased production of superoxide, inflammatory macrophage polarization and impaired cellular oxygenation.

Keywords: Precocious puberty. Genetic mutation. Metabolism. Methyl-CpG-binding protein 2. Metabolic syndrome.

1. INTRODUÇÃO

1.1 PUBERDADE FISIOLÓGICA

A puberdade é definida como o período biológico em que ocorre a transição da infância à fase adulta e que compreende diversas alterações físicas, hormonais e psicológicas, levando ao desenvolvimento de caracteres sexuais secundários e à capacidade reprodutiva do indivíduo (1,2). O eixo hipotálamo-hipófise-gonadal tem comportamento variável ao longo do desenvolvimento humano. Na fase pós-natal imediata há uma ativação inicial do eixo, período mais conhecido como "mini puberdade", cuja função biológica ainda é pouco conhecida (3). Após a "mini puberdade" há uma fase de inibição completa do eixo, que se prolonga por toda infância. Na puberdade, há um aumento da frequência e da amplitude da secreção de hormônio secretor de gonadotrofinas (GnRH), que, por sua vez, estimula a secreção de hormônio luteinizante (LH) e de hormônio folículo estimulante (FSH) na hipófise anterior. Estes hormônios, por sua vez, estimulam a maturação gonadal e a secreção de hormônios sexuais, que levam ao desenvolvimento de caracteres sexuais secundários (1,2). Ao longo do estudo dos fatores que promovem o início da puberdade e a ativação do eixo reprodutivo, diversos fatores estimuladores e inibidores têm sido identificados. Desta forma, a ativação do eixo puberal se dá quando há um aumento dos fatores ativadores do eixo reprodutivo e uma supressão dos fatores inibidores (4).

Do ponto de vista clínico, o início da puberdade se dá, de acordo com os critérios de Marshall e Tanner (5,6), em meninos por meio do aumento de volume testicular e em meninas pelo surgimento do broto mamário (2). Classicamente, se considera que a faixa de normalidade para o início da puberdade compreende o período entre 8 e 13 anos no sexo feminino e 9 a 14 anos no masculino (7). Não obstante, tem se observado que o início da puberdade tem sido mais precoce em todo o mundo e, em especial, em meninas afro-americanas (8). Um dos motivos que se atribui a essa maior precocidade é o aumento da prevalência de obesidade e outros distúrbios nutricionais.

1.2 PUBERDADE PRECOCE – DEFINIÇÕES E EPIDEMIOLOGIA

Puberdade precoce é caracterizada pelo surgimento de caracteres sexuais secundários antes dos 8 anos em meninas e antes dos 9 anos em meninos, associada ao aumento de velocidade de crescimento, aceleração da maturação óssea e elevação da concentração sérica de hormônios sexuais (1,2).

A incidência e prevalência de puberdade precoce diferem muito entre estudos de diferentes países. Um primeiro estudo dinamarquês que considerou puberdade precoce quando diagnosticada ou tratada em meninas até 9 anos e meninos até 10 anos identificou a patologia em 3 a cada 10.000 meninas e 0,4 a cada 10.000 meninos (9). Um outro estudo dinamarquês, ainda mais recente, avaliou dados populacionais por um período de 20 anos (1998 a 2017) e demonstrou que houve um aumento de prevalência de diagnóstico de puberdade precoce no período: de 9,2 para 14,2 a cada 10.000 meninas e de 0,9 para 1,0 a cada 10.000 meninos, se utilizasse o mesmo critério do estudo anterior (10). Um estudo epidemiológico espanhol avaliou especificamente PPC, seguindo os critérios mais aceitos (8 anos em meninas e 9 em meninos e valores séricos púbere de LH após teste de estímulo), encontrou uma prevalência média geral de 1,9 casos por 10.000 (sendo 3,7 por 10.000 em meninas e 0,046 por 10.000 em meninos) (11). Demonstrou-se, também, um padrão nítido de aumento da incidência ao longo do período estudado (1997 a 2009). Posteriormente, um estudo coreano, que coletou dados de 2004 a 2010, estimou a prevalência de PPC em 2010 de 5,6 por 10.000 meninas e 0,17 por 10.000 meninos(12). Observou-se, também, nesta casuística o aumento na incidência anual de PPC, de 0,33 a 5 por 10.000 meninas e de 0,03 a 0,12 por 10.000 meninos durante o período analisado (12). Em outro estudo, pesquisadores franceses analisaram os dados de meninas com puberdade iniciada com menos de nove anos de idade e meninos com menos de 10 anos, no período entre 2011 e 2013; e observaram uma incidência anual de PPC de 2,68 por 10.000 meninas e 0,24 por 10.000 meninos (13). Os estudos descritos divergiram muito de metodologia para coleta de dados, e, consequentemente, a prevalência e a incidência de puberdade precoce variou entre eles. Ainda assim, todos demonstram uma predominância do diagnóstico em meninas e uma tendência de aumento de incidência ao longo dos anos.

A puberdade precoce associa-se a risco aumentado de baixa estatura na vida adulta (secundária a maturação óssea acelerada) e a desfechos psicossociais

adversos (14). Além disso, o início precoce da puberdade tem sido associado a maior incidência na vida adulta de obesidade, hipertensão arterial, diabetes mellitus tipo 2, doenças cardiovasculares e cerebrovasculares, neoplasia de mama, de endométrio, de testículo e de próstata e mortalidade por todas as causas (15–17).

A puberdade precoce pode ser dividida em puberdade precoce dependente de gonadotrofinas ou puberdade precoce central (PPC), quando decorre da ativação prematura do eixo reprodutivo, e em puberdade precoce independente de gonadotrofinas ou puberdade precoce periférica (PPP), quando se dá pelo aumento da concentração de esteroides sexuais independentemente da ativação deste eixo (1,2).

Dentre as causas de PPC observam-se três distintos grupos. O primeiro é o das causas ditas orgânicas, isto é, que se caracterizam pela presença de lesões em sistema nervoso central (de origem congênita ou adquirida, sendo a lesão mais comum o hamartoma hipotalâmico) (18). O segundo grupo é o de causa idiopática. Este grupo representa classicamente a principal forma em meninas, podendo chegar a 90% dos casos de PPC, enquanto nos meninos é responsável por 25 a 60% dos casos (19,20). Já o terceiro grupo é constituído das causas genéticas. Previamente classificados como idiopáticos, nos últimos anos identificaram-se diversos genes associados ao surgimento de PPC familiar. Mutações ativadoras no gene que codifica a kisspeptina e seu receptor foram inicialmente descritas como possíveis causadoras de PPC (21,22). Subsequentemente, identificaram-se mutações inativadoras no gene *MKRN3* (gene codificador da proteína *makorin ring finger 3*) em diversas famílias com PPC familiar (23). Este gene constitui a principal causa de PPC genética atualmente conhecida, sendo posteriormente identificado também em alguns casos previamente classificados como esporádicos e idiopáticos (24).

Mais recentemente se identificaram mutações inativadoras do gene *DLK1 (Delta-Like 1 homolog)* como causa adicional de PPC genética por meio do estudo de PPC familial. As análises de ligação gênica e sequenciamento genômico completo permitiram a identificação de mutações que geravam perda de função deste gene, com padrão de herança autossômico dominante, porém de transmissão paterna (imprinting materno). De forma interessante, os pacientes com mutação têm uma prevalência aumentada de obesidade e síndrome metabólica, com duas pacientes do sexo feminino apresentando síndrome dos ovários policísticos (25,26).

Por fim, em 2023 foram descritas mutações raras no gene MECP2 em casos de PPC idiopática esporádica, apontando para um possível papel deste gene, que é expresso em camundongos em regiões hipotalâmicas importantes para a regulação do GnRH, sobre a regulação puberal (27).

1.3 PUBERDADE PRECOCE – FISIOPATOLOGIA E GENÉTICA

A kisspeptina foi descrita em 2003. Trata-se da proteína do gene *Kiss1,* que atua sobre os receptores *KISS1R* (também denominado GPR54) e que possui papel importante no início da puberdade (28). Estudos em roedores demonstraram que, ao longo do desenvolvimento puberal, houve um aumento no número de neurônios de kisspeptina e da expressão do *Kiss1* no tecido hipotalâmico (29). Os neurônios de kisspeptina se encontram em duas regiões: na área periventricular anteroventral (AVPV) e no núcleo arqueado (ARC) (30). Aqueles localizados no ARC também co-expressam neurocinina B (NKB) e dinorfina (Dyn) e são mais conhecidos como neurônios KNDy. Nestes neurônios, enquanto a NKB é estimulatória à sinalização via kisspeptina, a Dyn tem efeito inibitório sobre o eixo puberal (31) **(Figura 1)**.

Figura 1 - Localização dos neurônios de kisspeptina e ação sobre os neurônios de GnRH

FONTE – Produção do próprio autor.
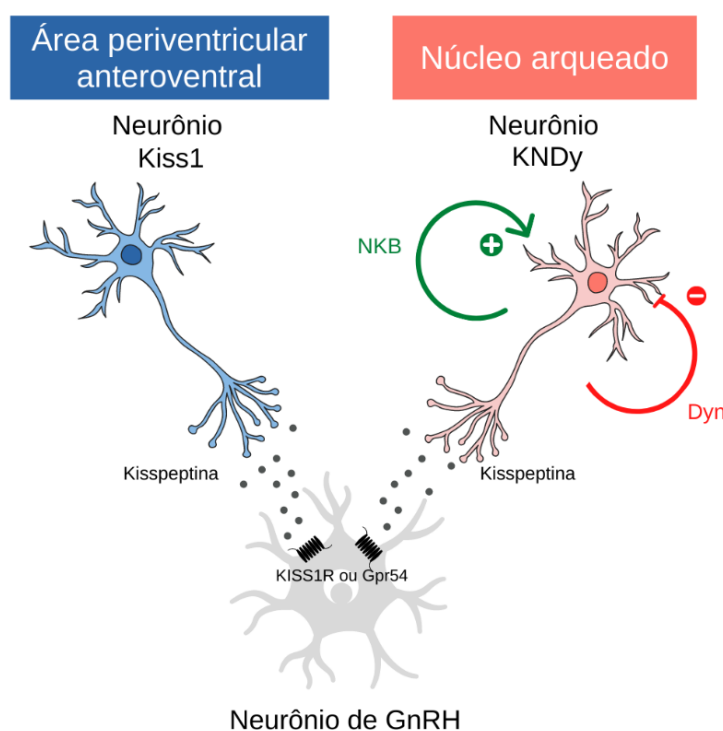
Em 2013, um novo fator foi adicionado a essa complexa regulação neuroendócrina de ativação central do eixo reprodutivo. Foram inicialmente descritas cinco diferentes famílias com puberdade precoce central familiar causada por mutações inativadoras do gene *MKRN3* (23). Tais mutações são herdadas com padrão de *imprinting* materno, ou seja, o fenótipo é herdado apenas quando herdado do alelo paterno, pois o alelo materno é silenciado. Atualmente, mutações no *MKRN3* são a principal causa de PPC de etiologia genética. Um trabalho recente compilou uma casuística de 716 casos de PPC e familiares afetados, provenientes de nove países diferentes e encontrou mutações inativadoras do *MKRN3* em 71 pacientes (9,9%) previamente considerados idiopáticos (32) **(Figura 2).**

Figura 2 - Representação da proteína MKRN3 e localização das mutações tipo frameshift (Vermelho), stop gain (amarelo) e missense (azul)(32)
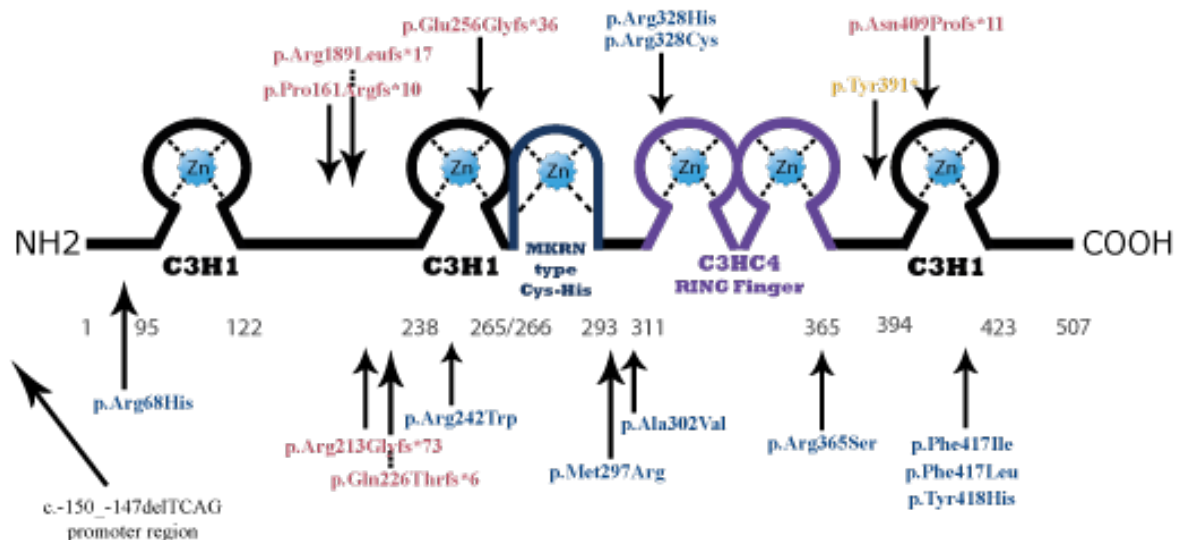
FONTE: Produção do próprio autor, publicada em 2021 (32)A proteína do MKRN3 é composta por 3 anéis C3H1, um domínio tipo MKRN Cys-His e um anel de zinco C3HC4. As flechas apontam para a região da mutação correspondente na proteína.

As concentrações de RNA mensageiro do Mkrn3 no hipotálamo de roedores são elevados durante o início da vida, e têm suas concentrações reduzidas durante a puberdade em regiões importantes para a ativação puberal, tais como o ARC e AVPV (33), sugerindo um efeito inibitório do MKRN3 sobre a puberdade – que foi perdido em pacientes que tinham mutações inativadoras deste gene – e, mais recentemente, demonstrou-se que isso se dá pois o *MKRN3* exerce papel inibitório sobre a região promotora de genes que estimulam a secreção pulsátil do GnRH, como o *KISS1* e a

TAC3 (34). O próprio *MKRN3* pode ser inibido através do microRNA miR-30, segundo achados de um estudo em camundongos (35). Desta forma, a fisiologia básica proposta pelos trabalhos mais recentes de ativação neuroendócrina da puberdade encontra-se resumida na **Figura 3**, adaptada de Maione L *et al* (36).

Figura 3 - Atividade do MKRN3 sobre neurônios de kisspeptina
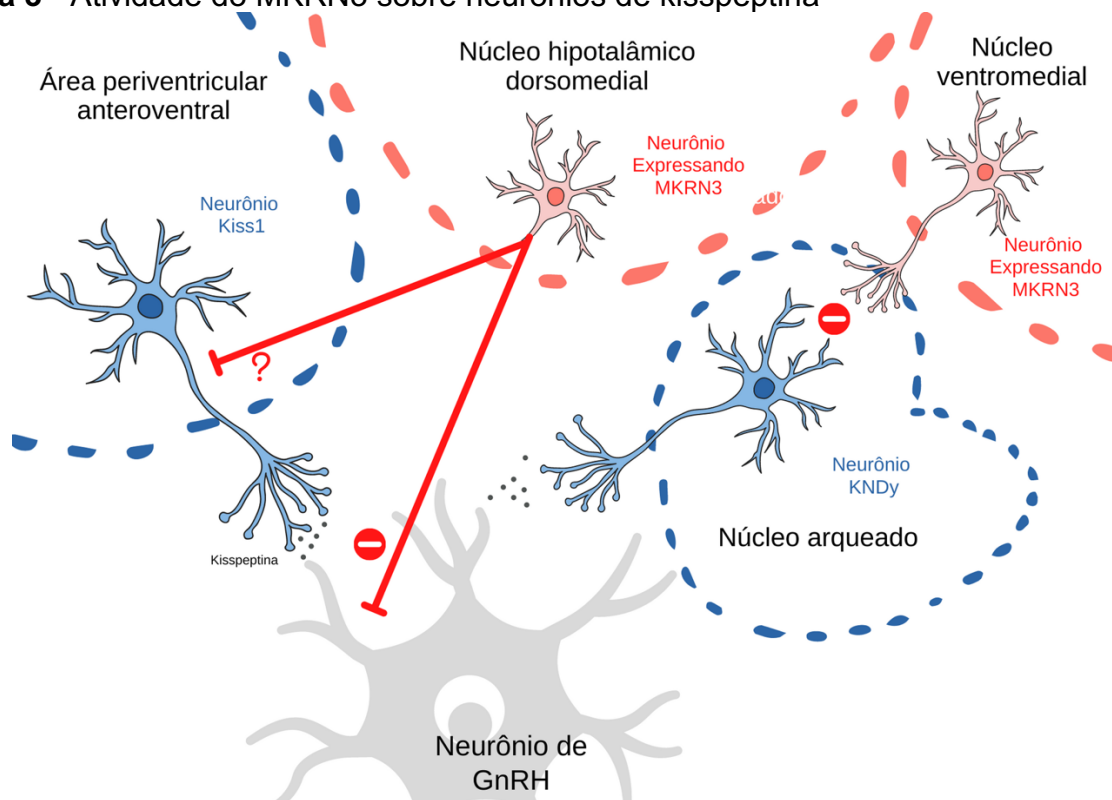
FONTE: Produção do próprio autor, adaptada de Maione L et al (36)

Em 2017 foram descritas mutações inativadoras no *DLK1* que levavam a PPC em 10 pacientes do sexo feminino de quatro famílias (25,26). Uma das mutações era um defeito complexo com deleção de aproximadamente 14 kb e duplicação de 269 pb. As outras 3 famílias possuíam 3 mutações do tipo *frameshift* distintas. Subsequentemente, em uma casuística da Espanha analisada em conjunto com grupo de pesquisa brasileiro, identificou-se uma mutação *de novo*, do tipo deleção (c.401_404 + 8 del) na região de *splicing* do *DLK1* (37). No estudo colaborativo, foi realizada a investigação clínica e genética de uma coorte de 444 indivíduos da Espanha (168 casos-índice), encontrando 5 mutações diferentes no *MKRN3* e a mutação descrita no *DLK1*. Além dessa variante patogênica, encontraram-se três outras variantes alélicas raras (p.Asn134=; g.-222 C>A e g.-223 G>A) em duas

meninas; no entanto, estas apresentavam níveis séricos de DLK1 normais (37). Posteriormente, encontraram-se casos de PPC causada por mutação no *DLK1* na Itália, na China e na França, sendo que nos dois primeiros países foram descritos meninos afetados pela primeira vez (38–40). As características clínicas e as mutações de todos os pacientes descritas até o momento na literatura estão representadas na **Tabela 1**.

Tabela 1 Características clínicas e metabólicas das pacientes com mutações inativadoras do *DLK1* descritas na literatura

Família (Origem)	Caso (Número)	Sexo (M ou F)	Idade na publicação (anos)	Início da puberdade (anos)	Idade da menarca (anos)	Altura final (cms) (Z)	IMC (kg/m^2) (Z)	Fenótipo adicional	Mutação (cDNA) (proteína)	Publicação
1 (Brasil)	1A	F	34	5	7	155,7 (-1,1)	29,2 (1,45)	SOP, infertilidade, DM2 (27a) e esteatose hepática	c.594_594delC p.Gly199Alafs*11	Dauber A et al (25)e Gomes L et al (26)
	1B	F	24	5	7	156,7 (-0,9)	27,2 (1,21)	SOP, infertilidade, DM2 (16a) e esteatose hepática		
2 (Reino Unido)	2A	F	29	5	11	146,2 (-2,6)	21,1 (-0,14)	---	c.810_810delT p.Val271Cysfs*14	
3 (Brasil)	3A	F	19	7	7	145,2 (-2,8)	13,2 (-6,2)	---	c.479_479delC p.Pro160Leufs*50	
	3B	F	56	7	9	137,8 (-4)	37,7 (2,08)	Intolerância à glicose e dislipidemia		
4 (Brasil)	4A	F	22	5,5	12	156,5 (-0,9)	29,2 (1,46)	Aumento % gordura	Rearranjo genômico complexo com deleção do exon 1 e da região 5`UTR / Proteína truncada	
	4B	F	21	5	12	159,7 (-0,4)	22 (0,14)	Aumento % gordura		
	4C	F	18	4,6	12	159,3 (-0,3)	33 (1,8)	Aumento % gordura e intolerância à glicose		
	4D	F	16	5,9	10,8	160,5 (-0,3)	22 (0,46)	Aumento % gordura		
	4E	F	63	ND	~9	154,0 (-1,3)	29,6 (1,44)	DM2, HAS, dislipidemia		
5 (Espanha)	5A	F	15,9	5,7	11,2	153,5 (-1,4)	17,6 (-1,2)	---	c.401_404+8del Proteína truncada	Montenegro L et al (37)
6 (China)	6A	M	10,4	9	---	143,5 * (0,65)	19,1 (0,89)	Hiperuricemia	c.479delC p.Pro160Leufs*50	Yuan G et al (40)
7 (Itália)	7A	M	9,1	8	---	153,8 * (1,3)	22,1 (1)	---	c.288_289insC	Palumbo S et al (38)

(cont.) 7	7B	F	6,2	6,2	---	134 (1,3)	20,83 (1,4)	---	p.Cys97Leufs*16	
Itália	7C	M	51,7	8	---	159,4 (-2,8)	22,67 (- 0,16)	Dislipidemia		
8 (França)	8A	F	5,5	4,5	---	114,5 (0,85)	(2,48)	---	c.372C>A p.Cys124X	Seraphim CE / Montenegro L et al (39)
9 (França)	9A	F	6	6	---	120 (1,3)	(0,9)	---	c.2T>G p.Met1Arg	

FONTE: Produção do próprio autor. * Não representa a altura final, mas sim a última avaliação de altura de pacientes em seguimento.

O *Dlk1* é expresso no hipotálamo de camundongos, o que sugere seu papel central na regulação do eixo puberal e sua expressão aumenta no hipotálamo destes camundongos da infância para a fase adulta. No entanto, a concentração sérica de Dlk1 nestes animais reduz no mesmo período (39,41). Tal padrão também é demonstrado na puberdade humana, havendo uma redução gradativa dos níveis de DLK1 sérico ao longo dos diferentes estadiamentos puberais (39).

Em 2023 foram descritas três mutações no gene *MECP2* em sete meninas com PPC, sendo que três delas possuíam também alterações neuro cognitivas (microcefalia e autismo). Mutações no gene *MECP2* classicamente causam síndrome de Rett, no entanto, nos casos descritos havia apenas a PPC, com ou sem alterações neurológicas leves (27). O gene Mecp2 também é co-expresso no hipotálamo de camundongos e de ovelhas, indicando sua possível interação com a regulação hipotalâmica do desenvolvimento puberal (27,42).

Na **tabela 2** estão representados todos os genes já descritos em associação com a puberdade precoce central em humanos.

Tabela 2 - Características dos genes descritos em associação à puberdade precoce central

Gene	Descriçã o (ano)	Localização cromossômic a	Herança	N na literatura	Tipo de mutação	Característi cas clínicas adicionais	Primeira descrição
KISS1R	2008	19p13.3	AD	1	Mutação ativadora	---	Teles M et al (21)
KISS1	2010	1q32.1	AD	3	Mutação ativadora	---	Silveira LG *et al.* (43)
MKRN3	2013	15q11.2	AD Imprinting materno	> 200 9 a 10,7% dos casos familiares	Mutações inativadoras	---	Abreu AP et al (23)
DLK1	2017	14q32.2	AD Imprinting materno	17	Mutações inativadoras	Obesidade e síndrome metabólica	Dauber A et al (25)
MECP2	2023	Xq28	Ligada ao X	7	Mutações inativadoras	Alterações neuro cognitivas	Canton A et al (27)

AD: autossômica dominante.

FONTE: Produção do próprio autor.

1.4 ASSOCIAÇÃO ENTRE METABOLISMO E PUBERDADE

A correlação entre puberdade e metabolismo tem sido cada vez mais elucidada na literatura médica. Estudos epidemiológicos demonstram uma clara correlação entre início mais precoce da puberdade e maior IMC, maiores níveis de insulina no jejum, diminuição de HDL e aumento de LDL e de triglicérides, ou seja, entre puberdade mais precoce e componentes da síndrome metabólica (15,44). Um estudo utilizando uma base de dados do Reino Unido (UK Biobank Study) mostrou que o início mais prematuro da puberdade em indivíduos caucasianos se associou a maior risco de angina, hipertensão arterial e diabetes mellitus tipo 2 (16). Estudos de GWAS (*genome wide association studies*) têm associado idades mais prematuras de menarca com diversos riscos cardiometabólicos (17,44).

Diversas hipóteses foram historicamente levantadas para explicar a associação entre obesidade/síndrome metabólica e puberdade. Uma das principais hipóteses é a do "peso crítico". Esta postula que haveria um efeito direto das adipocinas sobre a puberdade, em especial da leptina, pois esta é uma citocina sabidamente permissiva para a atividade dos neurônios hipotalâmicos produtores de GnRH (45). Como a leptina é produzida nos adipócitos e sua concentração sérica aumenta proporcionalmente ao aumento de peso e conteúdo de gordura corporal, estabeleceu-se um primeiro elo entre obesidade e início de puberdade (45).

Contribuiriam também a maior atividade da enzima aromatase no tecido adiposo em pacientes com sobrepeso/obesidade, convertendo andrógenos a estrógenos, a maior ativação do eixo do IGF-1 e resistência à insulina, assim como desreguladores endócrinos (46). Além disso, a própria puberdade precoce poderia predispor à maior obesidade, uma vez que o estradiol, por exemplo, se associa ao ganho de massa gorda.

Além disso, sabe-se que há envolvimento de vias associadas ao apetite central no desenvolvimento puberal. O gene *NPYR1* codifica o receptor para o neuropeptídeo Y (NPY) e antagoniza os efeitos inibitórios do neurotransmissor GABA sobre os neurônios de GnRH. Há evidências em modelos animais de papel importante desse antagonismo das vias inibitórias para o desenvolvimento da puberdade (47). Enquanto o neuropeptídeo Y, que é um fator orexígeno, inibe o desenvolvimento puberal, a leptina, que é uma adipocina que sinaliza o excesso de nutrientes estimula o GnRH

(48). Além disso, a ghrelina, que é um fator orexigênico liberado por células do fundo gástrico e sinaliza a deprivação energética, inibe a secreção de GnRH (49).

As concentrações séricas de leptina aumentam em meninas antes do início da puberdade e há um pico de leptina que precede o pico de gonadotropinas (50,51). Um estudo demonstrou uma correlação entre os níveis circulantes de leptina e a idade da menarca em meninas, demonstrando que para cada 1 ng/mL a mais de leptina a menarca se iniciava 1 mês mais cedo e que a cada 1 kg de gordura corporal a mais havia menarca 13 dias mais cedo (50).

A ação da leptina sobre as vias hipotalâmicas da puberdade não se dá diretamente sobre os neurônios de GnRH, já que estes não possuem receptores de leptina. Na realidade, esta ação se dá através dos neurônios de kisspeptina. Na deficiência de leptina em camundongos, demonstrou-se a redução da expressão de *Kiss1* no núcleo arqueado e a administração de leptina nestes modelos animais levou ao aumento da expressão de *Kiss1* (31). As evidências indicam que os neurônios KNDy de kisspeptina são altamente sensíveis ao status nutricional e aos níveis de leptina (52). Situações de deprivação energética importantes, como desnutrição ou anorexia, levam à menor expressão hipotalâmica de *Kiss1* nos neurônios KNDy, e a administração de kisspeptina, nestas situações, leva ao reestabelecimento do eixo gonadal (29). Quando à excesso de nutrientes, na obesidade, a resposta da expressão de *Kiss1* parece ser bifásica. Enquanto a obesidade no período pré puberal leva ao aumento da expressão de *Kiss1* e à precocidade sexual, na vida adulta há redução da expressão hipotalâmica deste gene (53). Apesar desse aparente efeito direto, há também modelos importantes apontando para que haja maior importância de um efeito indireto da leptina sobre os neurônios de kisspeptina, envolvendo outras vias, como as de glutamato ou GABA, por exemplo (53).

Em outra frente fisiológica, as vias neuroendócrinas da puberdade podem ser reguladas em função do status energético também pelos sensores intracelulares de energia, como o mTOR (alvo da rapamicina em mamíferos), a AMPK (proteína quinase ativada por AMP) e a SIRT1 (sirtuína) (54). Essas moléculas interagem de forma significativa com os neurônios KNDy de kisspeptina. Quando há excesso ou suficiência energética, há aumento dos níveis de mTOR e menor ativação de AMPK; enquanto na deprivação energética há ativação preferencial da AMPK e inibição do mTOR. Esta última situação leva à supressão do *Kiss1* e atraso puberal (53). De forma semelhante, na deprivação energética há aumento dos níveis da SIRT1 e ligação

desta à região promotora do *Kiss1,* de forma a inibir sua expressão (53). No início da puberdade, há redução da SIRT1, permitindo a ativação da via kisspeptina. Quando há obesidade e excesso de nutrientes, há uma redução mais precoce da SIRT1 e tendência à precocidade sexual(55).

Ainda no nível molecular, um estudo em camundongos fêmeas demonstrou o papel das ceramidas sobre o eixo reprodutivo no sistema nervoso central. Na obesidade, há aumento do conteúdo de ceramidas no sistema nervoso central, o que é permissivo para a puberdade, levando, portanto, à precocidade sexual. Este efeito das ceramidas for mimetizado com a ativação farmacológica da via das ceramidas e impedido quando se bloqueou por transfecção viral esta via. Vale destacar que esta via aparenta ser independente da via clássica de ativação do GnRH e se dá no núcleo paraventricular, com projeções da inervação simpática ovariana (56).

Polimorfismos em genes associados a obesidade, como o *LIN28B* e o *FTO* foram também associados com diminuição da idade da menarca (57).

Os mecanismos conhecidos de interação entre obesidade e puberdade estão resumidos na **Figura 4**.

Figura 4 - Regulação metabólica do eixo reprodutivo em condições de déficit e excesso de nutrientes.
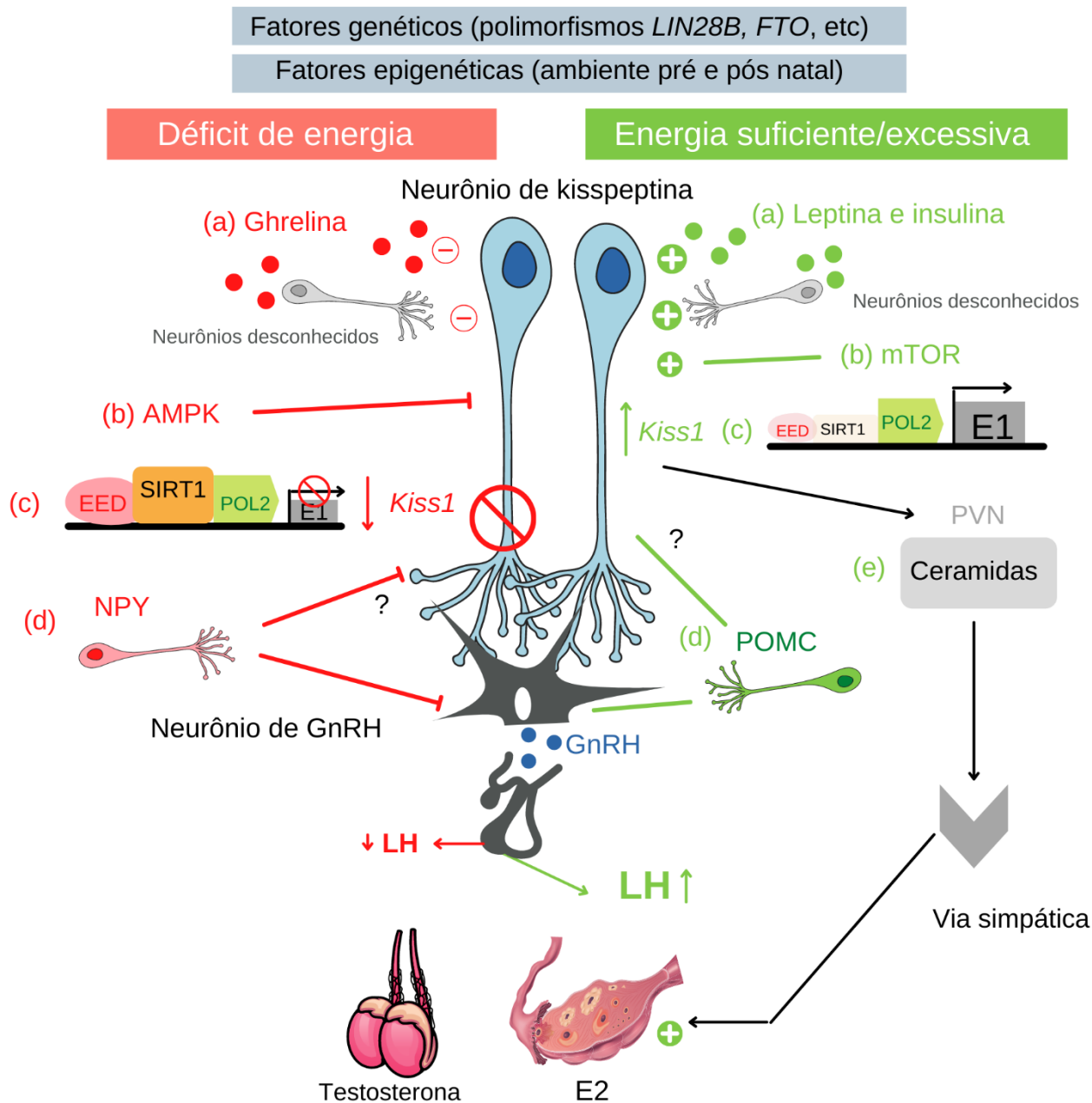
FONTE – Produção do próprio autor, adaptada de publicações anteriores (58,59). Resumo dos mecanismos de interação entre os sensores metabólicos e o eixo da puberdade. Quando há privação energética, há tendência há bloqueio da sinalização da puberdade via bloqueio da kisspeptina, que se dá através da sinalização da (a) ghrelina, (b) do aumento da concentração de AMPK, (c) da elevação da SIRT1, que bloqueia a região promotora do *KISS1 e* (d) da ação inibitória dos neurônios das vias orexígenas (NPY) sobre os neurônios de GnRH e possivelmente Kiss1. Já no excesso de nutrientes ou na suficiência energética, há permissividade à ativação da kisspeptina, através (a) da leptina e da insulina, (b) do aumento intracelular do mTOR, (c) da redução da SIRT1, levando a expressão da *Kiss1* e (d) de aferentes da via de saciedade (POMC) sobre os neurônios de GnRH e possivelmente Kiss1*. A via das ceramidas (e) é ativada por aferentes dos neurônios de kisspeptina no núcleo paraventricular

(VPN). O aumento de ceramidas nessa região leva a estímulo direto ovariano por uma via alternativa mediada por sinalização simpática. GnRH, hormônio liberador de gonadotropinas; mTOR, alvo da rapamicina em mamíferos; SIRT1, sirtuína; EED, cofator repressor membro da família polycomb; POL2, RNA polimerase 2; LH, hormônio luteinizante; FSH, hormônio folículo estimulante.

1.5 *DLK 1* – UM NOVO LINK ENTRE METABOLISMO E PUBERDADE

O *DLK1*, também conhecido como fator pré adipócito 1 (*PREF-1*) ou antígeno fetal tipo 1 (*FA-1*), é um ligante do tipo "*notch*" não canônico que participa de diversos processos intracelulares, sendo expresso em adultos em diversos tecidos, tais como, hipofisário, adrenal, pancreático e gonadal. Na fase embrionária também é expresso em adipócitos e tecido muscular (60).

A família de receptores do tipo "notch" se caracteriza por ser uma via altamente conservada entre as espécies, caracterizada por ter uma proteína transmembrana. Esta proteína tem uma porção extracelular mais longa ligada a uma porção extracelular menor por meio de uma ligação não covalente, com uma passagem na porção transmembrana e uma porção intracelular pequena. A ligação de uma proteína à porção extracelular do receptor "notch" gera proteólise e liberação do domínio intracelular, que fica disponível para ação nuclear, modulando a expressão gênica. Os ligantes ditos canônicos são proteínas de superfície de outras células e contém um domínio DSL (Delta, Serrate e Lag2) que interage com os receptores do tipo "notch", ativando-os, numa ligação que se denomina de "trans". Caso a ligação ocorra em "cis", isto é, o ligante esteja na superfície da mesma célula que contém o receptor, geram inibição competitiva da sinalização. Desta forma, cada célula pode enviar ou receber sinalizações, dependendo da quantidade de receptores e ligantes que expressam, mas não realizam ambas as funções ao mesmo tempo. Além disso, os ligantes e receptores do tipo "notch" sofrem glicosilação em regiões ricas em repetições do fator de crescimento epidérmico (EGF), o que pode aumentar ou diminuir a potência da sinalização intercelular, e podem sofrer também ubiquitinação, por meio de E3 ligases. Além disso, os ligantes podem ser clivados por enzimas do tipo ADAM e enzimas do tipo γ-secretase, liberando fragmentos do domínio extracelular. Estes podem exercer funções a distância, ligando-se aos receptores, e podem ativar ou inativar as vias de sinalização (61).

Os ligantes ditos não canônicos são aqueles que não possuem um domínio DSL. São um grupo heterogêneo de proteínas que exercem atividade sobre os receptores

do tipo "notch"(35). O DLK-1 é um dos primeiros ligantes não canônicos dos receptores "notch", sendo inicialmente descrito por seu papel importante na inibição da adipogênese. Apesar de não ter um domínico DSL, tem morfologia semelhante aos outros ligantes "delta-like", sendo também clivado pelas ADAMs. As evidências apontam que atua somente em "cis", gerando inibição das vias de sinalização "notch", provavelmente por competir com o sítio de ligação de outros fatores ativadores(61). Os mecanismos de regulação gênica e interação da proteína com os receptores NOTCH estão representados na **figura 5,** junto com a representação das primeiras mutações descritas em humanos (58)

Figura 5 - Gene e proteína DLK1, mutações descritas inicialmente no Brasil e interação com receptor NOTCH1
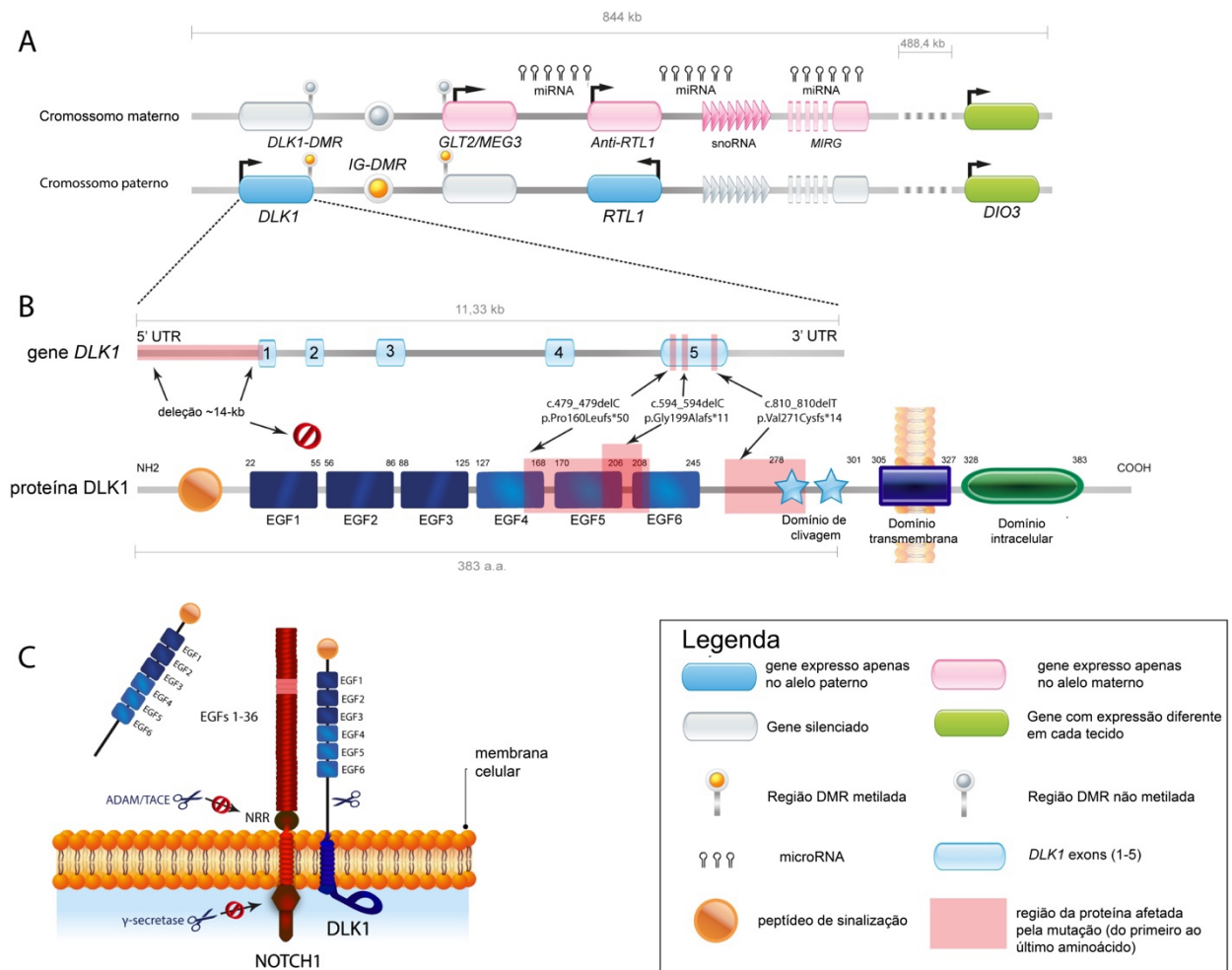
FONTE: Produção do próprio autor, publicada em (32)*(a)* – O locus *DLK-DIO3* está representado em ambos os alelos (paterno e materno). Enquanto o cromossomo paterno expressa os genes *DLK1* e *RTL1*, o cromossomo materno expressa o gene *GLT2/MEG3*, que é um gene não codificantes com um papel de supressor tumoral. Diversos miRNAs e snoRNAs são expressos também no cromossomo materno. O último gene no locus, *DIO3*, tem expressão diferencial de acordo com o tecido em que está localizado. A região de metilação DLK1-DMR está situada entre o intron 4 e

o exon 5 do gene *DLK1*. No entanto, a região DMR mais importante do locus é a IG-DMR, que é metilada durante a espermatogêneses no alelo paterno e não é metilada no alelo materno. A região IG-DMR metilada coordena o padrão de metilação e expressão dos outros genes: a metilação e expressão do *DLK1*, a expressão do *RTL1* e *DIO3* e a metilação e inativação do *MEG3*. **(b)** – O *DLK1* possui 5 exons. A figura representa o gene *DLK1* e os domínios correspondentes em sua proteína. As quatro diferentes mutações reportadas no Brasil estão representadas na figura. A deleção de 14 kb afeta o sítio de início de transcrição, gerando perda de transcrição do gene. As outras três mutações são do tipo *frameshift* e estão representadas no gene e na proteína correspondente, os quadrados vermelhos se iniciam no começo do frameshift e terminam no stop códon gerado, para representar o domínio afetado. As mutações p.Pro160Leufs∗50 e p.Gly199Alafs∗11 afetam as regiões dos EGFs 4, 5, e 6, que são justamente as regiões que mais interagem com os receptores NOTCH. Já a mutação p.Val271Cysfs∗14 está localizada após o EGF 6, mas gera um stop codon prematuro justamente na região de clivagem da proteína. **(c)** – Essa figura representa a interação da proteína do DLK1 (tanto na forma transmembrana quanto na porção extracelular solúvel) e o receptor NOTCH1. A interação do DLK1 com o receptor NOTCH1 inibe de forma competitiva a sinalização NOTCH, pois a interação do DLK1 com o receptor não expõe a região NRR para ser clivada pela enzima ADAM/TACE, logo, o domínio intracelular tampouco é clivado pela γ-secretase. A interação se dá entre os EGFs 11, 12 e 13 do NOTCH1 (representados em vermelho claro) com os EGFs 4, 5 e 6 do DLK1 (representados em azul claro). kb = kilobase; miRNA = microRNA; snoRNA = RNA pequeno e nuclear; *DLK1* = homólogo de delta-like 1; *GLT2/MEG3* = gene trap locus 2/maternally expressed gene 3; *RTL-1* = retrotransposon-like 1; *DIO3* = Dediodinase tipo 3 de iodotironina; *DLK1-DMR* = região diferencialmente metilada do *DLK1*; *IG-DMR* = região intergênica diferencialmente metilada; *MIRG* = gene contendo miRNA; UTR = região não traduzida; EGF-like = fator de crescimento semelhante ao da epiderme; a.a. = aminoácidos; NRR = região regulatória negativa.

O *DLK1* é um inibidor da adipogênese, pois inibe a diferenciação de pré-adipócitos em adipócitos maduros (62). Ele é considerado um marcador de pré-adipócitos. As concentrações séricas maternas de DLK1 tendem a se elevar ao longo da gestação, permanecendo elevadas durante a fase neonatal e posteriormente decrescendo ao longo da vida, voltando a aumentar apenas se houver gestação e/ou amamentação. A origem do DLK1 durante a gestação é fetal, e os níveis são maiores ao final da gestação. Sua função neste período parece ser de inibir, na mãe, a adipogênese e facilitar a lipólise, de forma a priorizar o aporte de nutrientes ao feto. Sua dosagem sérica se mostrou eficaz em diferenciar os fetos pequenos para idade gestacional saudáveis dos fetos patologicamente pequenos (63).

O *DLK1* tem participação importante também em praticamente todos os componentes da síndrome metabólica. Em indivíduos com diabetes mellitus tipo 2 foi demonstrada desregulação em células de ilhotas pancreáticas no *locus* DLK1-MEG3 (64). Na realidade, o DLK1 é fundamental para diferenciação de células ductais pancreáticas em células β e para produção de insulina. Isto se dá de duas formas, de maneira simplificada: os níveis elevados de DLK1 na embriogênese ativam a via MAPK, gerando translocação núcleo-citoplasmática de FOXO1 e PDX1 e diferenciação em células β e síntese de insulina; além disso ativa a via Akt e facilitam a secreção de insulina (65). A hiper expressão de *Dlk1* em modelos animais melhora a sensibilidade a insulina, com redução dos estoques de gordura branca, aumento dos níveis de GH e redução de esteatose hepática (66). Os modelos de roedores *knock-out* para *Dlk1* apresentam hipertrigliceridemia, resistência à insulina, aumento de colesterol e ácidos graxos livres circulantes (67).

Um estudo realizado no Reino Unido em 2014 utilizou roedores para avaliar a função do *Dlk1* e sua relação com a esteatose hepática (66). Por meio de roedores transgênicos, conseguiram simular um modelo de hiper expressão de *Dlk1*; os camundongos transgênicos adultos apresentavam níveis circulantes de Dlk1 de 4 a 7 vezes maiores que os níveis normais. Em seguida, alimentaram os animais com uma dieta rica em gordura, a fim de induzir esteatose hepática. Os animais com hiper expressão de *Dlk1* tiveram menor ganho de gordura branca, de forma significativa, e seu tecido adiposo apresentou um perfil favorável em relação à sensibilidade à insulina. Os níveis de adiponectina permaneceram iguais nos dois grupos, no entanto o grupo com hiper expressão de *Dlk1* apresentou níveis menores de leptina, compatível com o menor volume de gordura branca. Secundariamente, aumentaram

ainda mais o aporte calórico dos animais e utilizaram camundongos *ob/ob,* isto é, deficientes de leptina, de forma que os dois grupos ganharam tecido adiposo branco. Mesmo ganhando gordura, os camundongos *ob/ob* com excesso de Dlk1 não desenvolveram esteatose como no grupo com níveis normais de Dlk1 (40). Sendo assim, o excesso de Dlk1 não apenas previne o ganho de gordura e favorece a lipólise, como também dificulta o surgimento de doença gordurosa hepática não alcoólica **(Figura 6)** (66).

Figura 6 - Efeitos do excesso e da deficiência de *DLK1* obtidos de modelos animais

Excesso de DLK1

- Menor massa de tecido adiposo branco
- Maior lipólise
- Melhor sensibilidade à insulina
- Menor taxa de esteatose hepática
- Aumento dos níveis de GH

- Maior quantidade de tecido adiposo branco
- Hipertrigliceridemia
- Resistência à insulina
- Maior grau de esteatose hepática
- Elevação de colesterol

Deficiência de DK1
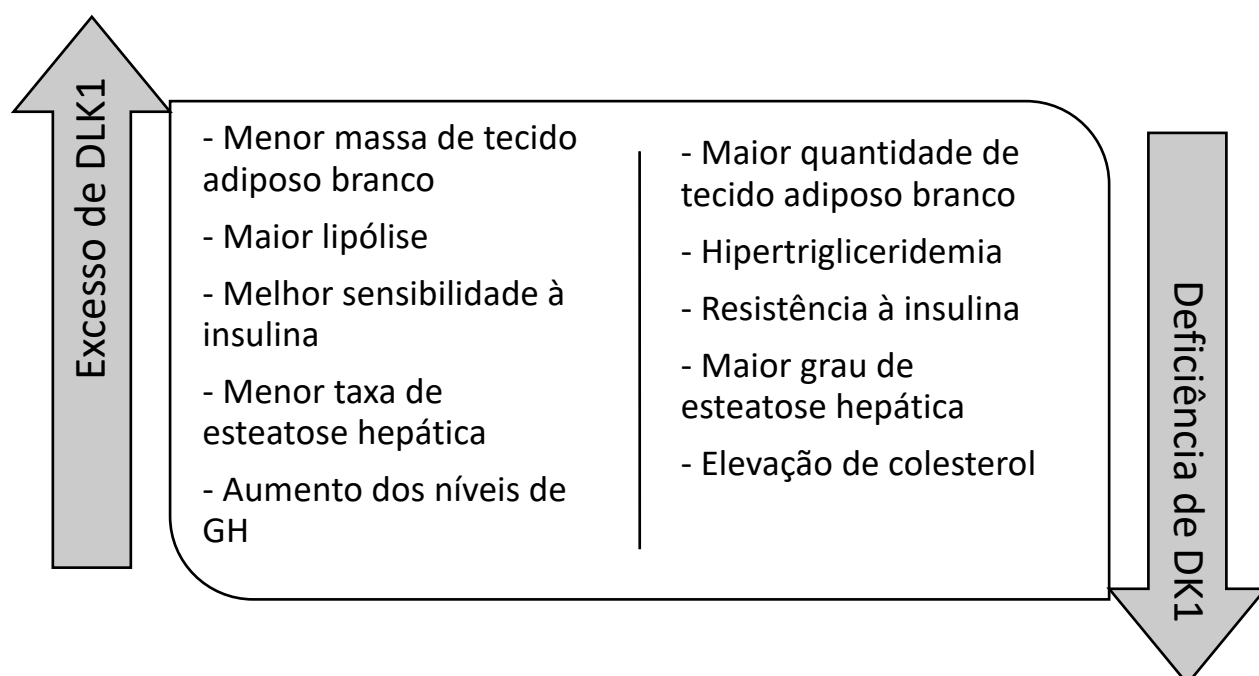
FONTE: Produção do próprio autor.

As mutações descritas em 2017 no *DLK1* que levaram a puberdade precoce central em dez indivíduos do sexo feminino eram acompanhadas mais frequentemente de obesidade ou sobrepeso (60%), diabetes mellitus tipo 2 (30%), e dislipidemia (50%) quando comparadas a um grupo de 20 mulheres adultas tratadas para PPC, apontando para que o *DLK1* seja um novo elo entre metabolismo e puberdade precoce (25). Estas prevalências de síndrome metabólica também são significantemente maiores que nas coortes de pacientes com PPC idiopática tratadas descritas na literatura (68). Posteriormente, diversas outras mutações no *DLK1* foram

descritas na literatura. As mutações descritas até o momento acometem apenas a porção extracelular da proteína. Os exons mais comumente afetados, 4 e 5, são responsáveis pelos EGFs 4 a 6, que constituem também a porção clivada extracelular que tem circulação sistêmica. Esta região é a principal responsável pela interação com os receptores NOTCH **(Figura 7).**

Figura 7 - Mutações descritas e suas posições no gene e na proteína DLK1
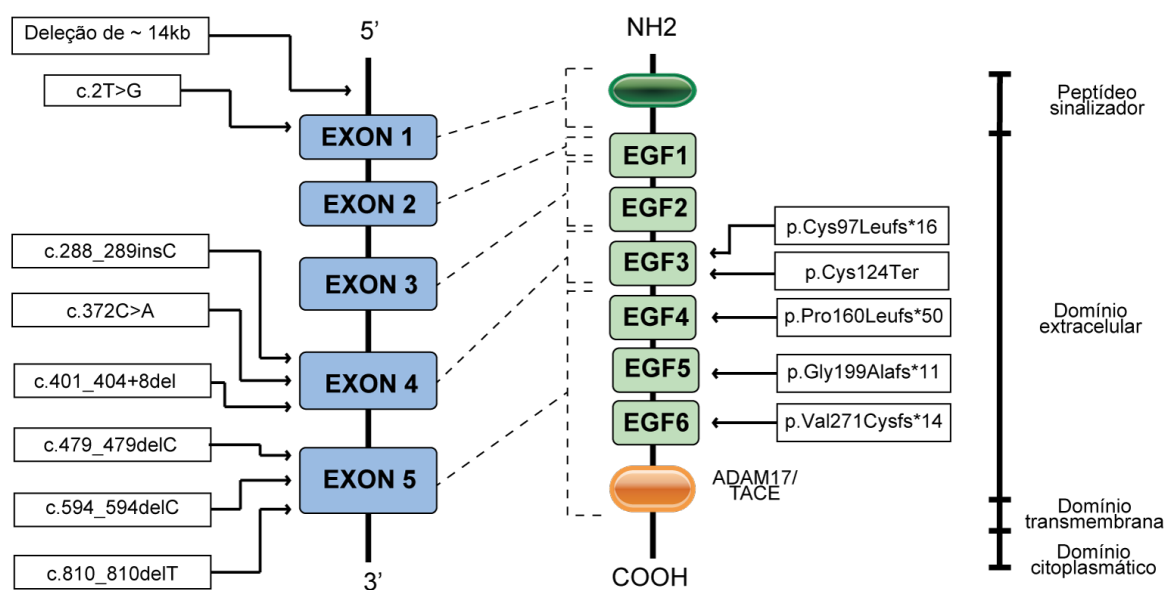
FONTE: Produção do próprio autor, adaptado de Palumbo S et al (38). À esquerda as mutações estão ligadas com uma flecha ao seu respectivo exon. As duas primeiras mutações levam à ausência de transcrição da proteína: a primeira afeta a região promotora do gene, a segunda leva à perda do códon de início. As linhas tracejadas indicam qual região da proteína DLK1 cada exon codifica. À direita, as mutações estão ligadas à proteína DLK1, excetuando-se as duas primeiras que não geram transcrição.

Vale destacar que o envolvimento do *locus* DLK1-DIO3 com fenótipo metabólico também é notado na síndrome de Temple. O próprio *locus* DLK1-DIO3 é localizado no cromossomo 14 (14q32.2), em região crítica para a síndrome. A síndrome de Temple é uma síndrome com defeito de imprinting, caracterizada por baixa estatura, hipotonia neonatal, mãos e pés pequenos. Além disso, 80-90% dos casos descritos tiveram precocidade sexual, bem como possuem maior risco de obesidade (11-49%), dislipidemia (10-23%) e DM2 precoce (11-20%) (69–71).

A porção extracelular do DLK1 pode ser dosada no soro. Um estudo dinamarquês, em 2019, descreveu uma correlação positiva entre os níveis séricos de DLK1 e o IMC, o percentual de gordura corporal, o HOMA-IR e o ADIPO-IR (72,73).

O MKRN3 também foi dosado no sangue em um estudo turco recente. Observou-se, também, menores níveis de MKRN3 circulante em indivíduos com PPC e obesidade. Embora seja um relato inicial, corrobora para a evidência de interação dinâmica entre fatores puberais e fatores metabólicos (74).

Na SM, já é bem descrita a existências de um perfil inflamatório sistêmico, com ativação preferencial de macrófagos pela via clássica (M1), e infiltração destes em estruturas em "coroa" ao redor dos adipócitos, associado a aumento de citocinas inflamatórias sistêmicas (75). Além disso, a SM também se associa a disfunção mitocondrial sistêmica e maior estresse oxidativo, com produção de radicais livres e superóxido (76). Não há ainda estudos na literatura sobre o perfil inflamatório e metabólico de pacientes com DLK1 não detectável.

2. OBJETIVOS

1- Caracterização clínica das alterações metabólicas de PPC de causa monogênica

2- Comparação das concentrações séricas de DLK1 em pacientes com PPC com e sem anormalidades genéticas

3- Descrição dos efeitos metabólicos da mutação do gene *DLK1* sobre a função de adipócitos

4- Avaliação da função mitocondrial de leucócitos e do perfil de ativação de macrófagos em pacientes com mutações do *DLK1*

3. MÉTODOS

3.1 CONSIDERAÇÕES ÉTICAS

Este estudo foi conduzido de acordo com princípios éticos seguindo as orientações contidas na declaração de Helsinki e pelos termos descritos pela Portaria 196/96 do Conselho Nacional de Saúde. O projeto foi aprovado pela Comissão de Ética para Análise de Projetos de Pesquisa (CAPPesq- HCFMUSP), CAAE: 18984419.2.0000.0068, conforme o parecer 3.552.804. Consentimento por escrito foi obtido de todos os pacientes ou pais/tutores, antes que os procedimentos de pesquisas fossem iniciados. Os achados de defeitos genéticos relacionados às doenças em investigação foram informados aos pacientes e/ou seus responsáveis legais, que terão aconselhamento genético e seguimento conforme o recomendado pela Sociedade Brasileira de Genética Médica no projeto Diretrizes do Conselho Federal de Medicina.

3.2 PACIENTES

Foram analisados dados 243 pacientes brasileiros com diagnóstico clínico e laboratorial de PPC acompanhados no Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP) ou referenciados de outros serviços (UNICAMP, Ribeirão Preto, e outras instituições) para estudo genético. Adicionalmente, contamos com colaboração internacional com pacientes de múltiplos centros espanhóis, com inclusão de 444 pacientes, e de casuística francesa com 121 casos índice.

Inclusão:
- Pacientes com início de puberdade segundo os critérios de Marshall e Tanner antes dos 8 anos no sexo feminino e antes dos 9 anos no sexo masculino
- Diagnóstico laboratorial de puberdade precoce central
  - LH basal > 0,3 UI/mL e/ou
  - LH pós estímulo com análogo de GnRH > 8 UI/mL
- Avanço de idade óssea segundo os critérios de Greulich e Pyle

Exclusão
- PPC de causa orgânica conhecida

- Lesões na ressonância magnética que sabidamente se associam a PPC orgânica (hamartoma, glioma de vias ópticas, entre outras já descritas na literatura)
- Pubarca ou Telarca precoce isoladas

3.3 COLETA DE DADOS CLÍNICOS

A coleta de dados clínicos se deu forma retrospectiva e prospectiva, compreendendo os casos de pacientes que realizaram seguimento e/ou tratamento no grupo de Puberdade Precoce nos últimos 20 anos. Todos os casos novos e os casos em seguimento atual no ambulatório de Puberdade Precoce, acrescidos dos casos referenciados de outros serviços foram acompanhados clínica e laboratorialmente. Os dados clínicos e laboratoriais resumidos avaliados na coorte brasileira estão representados na **Tabela 3**. O Z escore do IMC foi obtido das tabelas do CDC por ser mais adequado para avaliar obesidade em crianças e adolescentes. Valores entre 1 e 2 caracterizam sobrepeso, valores acima de 2 caracterizam obesidade e valores acima de 3 obesidade grave.

Tabela 3 - Características clínicas e metabólicas de 243 pacientes com PPC

	DLK1 (N=9)	Idiopática (N=226)	MKRN3 (N=8)	Total (N=243)	p
Apresentação inicial					
Z do IMC	---	0,86 (0,93)	0,87 (0,81)	0,87 (0,92)	0,58
Sobrepeso n (%)	---	71 (31,5%)	2 (25%)	73 (30%)	--
Obesidade n (%)		20 (8,8%)	1 (12,5%)	21 (8,6%)	--
Glicemia de jejum (mg/dL)	---	86,76 (8,22)	86,5 (6,36)	86,84 (8,02)	0,88
A1c (%)	---	5,28 (0,27)	---	5,31(0,31)	
Glicemia de jejum alterada e/ou A1c na faixa de pré diabetes n (%) *	---	4 (1,7%)	1 (12%)	5 (2%)	
Colesterol total (mg/dL)	---	150,92 (24,84)	169 (1,41)	152,62 (24,79)	0,24
LDLc (mg/dL)	---	83,95 (23,99)	102 (1,41)	85,6 (23,94)	0,25

HDLc (mg/dL)	---	48,53 (10,89)	42,5 (14,85)	48,37 (10,85)	0,66
TG (mg/dL)	---	89,1 (44,54)	123 (74,9)	90,4 (45,1)	0,57
Idade da menarca (anos)	10,7 (2,3)	11,2 (1,7)	11,7 (1,1)	11,2 (1,7)	0,65
Z do Peso ao Nascimento	0,14 (0,92)	-0,09 (1,34)	-0,56 (0,94)	- 0,1(1,31)	0,68
Z do Comprimento ao Nascimento	0,23 (1,23)	-0,71 (1,16)	-1,83 (0,58)	-0,7 (1,18)	0,07
Após o fim do tratamento					
Z da altura	0,95 (0,21)	1,22 (1,14)	2,07 (0,8)	1,23 (1,12)	0,39
Z do IMC fim do tratamento	0,74 (0,4)	0,89 (0,89)	1,43 (0,66)	0,9 (0,88)	0,45
Sobrepeso n (%)	1/4 (25%)	43/172 (25%)	2/8 (25%)	46/184 (25%)	---
Obesidade n (%)	0/4 (0%)	13/172 (7,6%)	1/8 (12,5%)	14/184 (7,6%)	---
Z IMC final do tratamento – Z IMC início do tratamento	-0,363 (0,634)	0,083 (0,548)	0,450 (0,566)	0,080 (0,557)	0,116

Dados apresentados na forma MÉDIA (DESVIO PADRÃO) ou n (%), conforme apropriado. As comparações entre 3 grupos foram realizadas pelo método ANOVA e as entre 2 grupos pelo teste T de Student. * Glicemia de jejum alterada – entre 100 e 125 mg/dL e/ou HbA1c entre 5,7 e 6,4%.

4.4 ANÁLISE GENÉTICA

DNA genômico foi isolado de leucócitos periféricos em todos os pacientes utilizando a metodologia padrão. Todas as regiões codificadoras do gene MKRN3 (GenBank NM_000015.10) e do gene *DLK1* (GenBank NM_003836) foram amplificadas por PCR seguido de sequenciamento automático dos produtos seguindo o método de Sanger para todos os pacientes. A análise de segregação familiar utilizando o método de Sanger também foi realizada.

3.5 AVALIAÇÃO HORMONAL

Para confirmação da puberdade precoce central foi dosado o LH basal, sendo sugestivo de ativação central do eixo com valores ≥ 0,6 U/L pelo método IFMA (ensaio

imunofluorométrico) ou ≥ 0,3 U/L pelo método ECLIA (ensaio eletroquimioluminescência) (2). Os pacientes com LH basal pré puberes realizaram estímulo com análogo de GnRH depot (acetato de leuprorrelina 3,75 mg sc ou im), com dosagens de LH e FSH após 60 minutos. Foram considerados para diagnóstico os valores > 6,9 U/L para o sexo feminino e > 9,6 U/L para o sexo masculino, pelo método IFMA, ou > 5 U/L para ambos os sexos, pelo método ECLIA (2).

3.6 DOSAGEM DE DLK1 SÉRICO

Medidas dos níveis séricos de DLK1 em pacientes com PPC foram realizadas utilizando um método de ELISA (IBL-America, Minneapolis, MN, USA). Os níveis séricos em humanos saudáveis oscilam entre 0,4 a > 2,5 ng/mL. O limite inferior de detecção do ensaio é < 0,4 ng/mL com uma variabilidade intra-ensaio de 5% e variabilidade inter-ensaio média de 1,8%. Os valores de DLK1 foram correlacionados com as etapas do desenvolvimento puberal, com a etiologia da PPC e com as características antropométricas e metabólicas.

3.7 ANÁLISE DE COMPOSIÇÃO CORPORAL

A avaliação de composição corporal e obtenção de medidas de massa magra, massa livre de gordura, massa musculo esquelética, massa gorda e percentual de gordura corporal foram obtidas utilizando um aparelho de bioimpedância (InBody 720 Biospace Co, Ltd, Seoul, Korea).

3.8 TOMOGRAFIA DE ABDOMEN PARA QUANTIFICAÇÃO DE GORDURA VISCERAL E TOMOGRAFIA DE CORONÁRIAS

A avaliação de composição corporal foi complementada em pacientes com mutações do *DLK1* com TC de abdômen para quantificação de gordura visceral no Instituto do Coração da FMUSP. Adicionalmente, foi realizada a TC com escore de cálcio de coronárias para avaliação de complicações macro vasculares metabólicas. A TC de abdômen foi avaliada por um único radiologista. Foram adquiridas imagens de tomografia com os pacientes na posição supina e os braços acima da cabeça, com obtenção de cortes de 10 mm em níveis L4-L5 (Helical Picker PQ 5000, Cleveland,

OH, USA). As imagens obtidas foram analisadas através do *software open source* Osirix Imaging Software (Pixmeo, Switzerland, v3.9). A gordura abdominal visceral (VAT) e a gordura abdominal subcutânea (SAT) foram obtidas através da demarcação no software, utilizando uma faixa de atenuação de -150 a -50 Unidades Hounsfield (UH) para caracterizar o tecido gorduroso (77,78).

Adicionalmente, na mesma ocasião da TC de abdômen, foram adquiridos cortes de coronárias. A imagem foi obtida em cortes finos de 2,5 mm, em sincronia com os batimentos de acordo com as medidas de eletrocardiograma, com rotação de 0,4 segundos e reconstrução temporal de 6 aquisições por segundo (79). A avaliação foi realizada por um único radiologista, e o escore de Agatston foi aplicado (80,81). Qualquer valor de escore de cálcio coronariano (CAC) acima de 0 foi considerado positivo, e os resultados foram classificados de acordo com os intervalos 0; 0 a 100 e > 100 unidades de Agatston (UA).

3.9 ANÁLISE DE CÉLULAS DE SANGUE PERIFÉRICO DE PACIENTES COM E SEM MUTAÇÃO DO *DLK1*

Amostras de sangue de 5 pacientes com mutações inativadoras no gene *DLK1* e 5 controles saudáveis pareados por média de idade e de IMC foram obtidas em tubos de coleta com EDTA. O sangue foi diluído em partes iguais com Dextran 2% (Sigma-Aldrich, USA) em solução salina e incubado por 20 minutos a temperatura ambiente. Realizou-se centrifugação a 700x G por 30 minutos para separação de plasma, polimorfonucleares e granulócitos. O material foi submetido a diferentes análises:

a) **Citometria de fluxo –** As frações celulares foram lavadas em PBS e foram coradas com 200nMMitoTrackerGreen (Invitrogen, Carlsbad, CA), 50nM MitoTrackerDeepRed (Invitrogen), e MitoSOX(5mM). Após o período de incubação, foram imediatamente analisadas em máquina de citometria de fluxo FACSCanto com o software BDFACSDiva (BDBiosciences, SanDiego, EUA) e foram processadas usando o software Flow-Jo (FlowJoLLC, AshlandOR, EUA).

b) **Diferenciação de macrófagos –** Foi realizada polarização de macrófagos alternativamente ativados (M2 Mθ), incubados com GM-CSF (25ng/mL) e

IL-4(10ng/mL) por 9 dias e submetidos a marcação para citometria de fluxo **(Anexo 1)**

c) **Diferenciação de pré adipócitos** – Foram utilizadas células 3T3-L1 (ABCAM, BOS, EUA) submetidas a um protocolo de diferenciação de adipócitos (Anexo2). Metade das células foram tratadas com soro de pacientes controle e metade com soro de pacientes com mutação do *DLK1*. As amostras foram coradas com Oil Red (ab150678, ABCAM, EUA) e analisadas por microscopia eletrônica.

d) **Expressão gênica em pré adipócitos** – A análise de expressão gênica foi realizada por RT-qPCR. A extração do RNA total foi feita pela técnica fenol-clorofórmio e realizou-se a confecção do cDNA (confeccionado a partir de 2000ng de RNA de todas as amostras).

e) **Avaliação de respiração celular in vivo** – Aferição da taxa de consumo de oxigênio (OCR) e da taxa de acidificação extracelular (ECAR) foram realizadas através da plataforma Seahorse XFe96 Analyzer (Agilent, Santa Clara, CA, EUA). Para tanto, foram extraídos os PBMCs do sangue, mantidos em cultura por 24 a 48 horas em meio próprio (PM-1, ZenBio), subsequentemente diferenciados por 7 dias em meio contendo HEPES (pH 7,4), 10% FBS, biotina, insulina, dexametasona, IBMX e agonista de PPARgama, seguidos de mais 7 dias de manutenção em meio de cultura de adipócitos. As células serão lavadas e 500 microL será adicionado em cada poço do aparelho. Será realizada a avaliação *real time* da OCR e da ECAR, permitindo avaliação da respiração mitocondrial e da glicólise. Indivíduos com obesidade, por exemplo, tem resposta atenuada da OCR ao isoprotenerol (82).

3.10 ANÁLISE ESTATÍSTICA

Os resultados dos dados clínico-laboratoriais para variáveis categóricas são expressos como valores numéricos (porcentagens) e, para variáveis contínuas, como medianas associadas ao intervalo interquartil. Comparações entre os diferentes grupos foram realizados pelos testes de qui-quadrado para variáveis categóricas e testes não paramétricos de Kruskal-Wallis ou teste T para variáveis contínuas. Em casos selecionados, foi realizada reamostragem por *bootstrapping* quando puder

acrescentar acurácia. Análises de correlação por regressão linear simples foram realizadas quando apropriado. Toda análise estatística foi realizada na plataforma R x64 (v 4.1.0) ou utilizando os pacotes seaborn, scipy, statsmodels, pandas do Python (v. 3.11). Um p <0,05 foi considerado estatisticamente significativo, a menos que se indique de outra forma.

4. RESULTADOS

4.1 – INTRODUÇÃO DA COLETÂNEA DE ARTIGOS

Foram selecionados quatro artigos de minha autoria ou coautoria publicados em revistas científicas internacionais e considerados mais relevantes para a presente tese. Os artigos científicos serão apresentados na ordem de ano da publicação. A lista com os demais artigos publicados no período do doutoramento encontra-se no **Anexo 4.**

O primeiro artigo intitula-se **"Delta-like 1 homolog genetics and its emerging role in human puberty"** e se trata de um trabalho de revisão publicado na revista *"Current Opinion in Endocrine and Metabolic Research"* em 2020. O artigo descreve o funcionamento da via NOTCH e do DLK1 enquanto inibidor desta, resume os achados das mutações em humanos com puberdade precoce central, seu impacto na proteína e função do DLK1 e as interações com aspectos metabólicos.

Já o segundo artigo*, "Genotype–phenotype correlations in central precocious puberty caused by MKRN3 mutations"*, publicado no *"Journal of Clinical Endocrinology and Metabolism"* em 2021 descreve a maior casuística de análise genética do MKRN3 realizada em um centro até a presente data, e descreve as características das mutações, incluindo análise computacional de conformação proteica e estabilidade energética das proteínas resultantes de variantes missense. Descreve-se, também o fenótipo da PPC causada pelo MKRN3, e conclui-se que esse é indistinto do fenótipo da PPC idiopática. Descreve-se, também a ausência de diferenças metabólicas significativas, e o IMC médio semelhante com o das causas idiopáticas. Conclui-se, portanto, que o fenótipo associado a SM é apenas encontrado em excesso nas mutações do *DLK1*, e não nas do MKRN3. O artigo foi um dos 10% mais lidos no ano de 2021 e foi incluído no anuário de artigos relevantes da sociedade europeia de pediatria (ESPE) **(Anexo 3).**

O terceiro artigo intitula-se *"Congenital and acquired mechanisms implicated in the etiology of central precocious puberty"* e trata-se de uma revisão publicada na *"Endocrine reviews"* em 2023. De especial interesse para a presente tese são as sessões em que se descrevem as interfaces entre a puberdade e seus aspectos metabólicos e a sessão sobre o DLK1, que inclui os dados atualizados até a data da apresentação sobre a casuística acompanhada pelo grupo na presente tese.

Por sua vez, o quarto artigo, chamado de **"Familial central precocious puberty due to DLK1 deficiency: novel genetic findings and relevance of serum DLK1 levels",** publicado no *"European Journal of Endocrinology"* também em 2023, descreve duas mutações causadoras de PPC inéditas no *DLK1*. Adicionalmente, foram dosadas em amostras de 209 pacientes em diferentes estágios da puberdade os níveis de DLK1 sérico e foi analisada sua relação com o IMC, a idade, o tipo de puberdade e o estadiamento puberal. Encontrou-se um padrão de queda dos níveis de DLK1 entre os primeiros estádios puberais e o último. Este dado foi confirmado e concordante com a dosagem de Dlk1 em camundongos, como descrito no artigo.

Finalmente, o artigo 5, que está em fase de submissão, avaliou 6 pacientes com mutação do *DLK1* comparadas a 6 controles saudáveis pareadas por idade e IMC. Demonstramos que a função mitocondrial das células de pacientes com a mutação do gene *DLK1* está prejudicada, com maior formação de superóxido (SOX). O plasma sem DLK1 das pacientes afetadas foi usado para diferenciação de adipócitos a partir de células progenitoras, e resultou na formação de tecido adiposo com maior grau de diferenciação de adipócitos e maiores gotículas lipídicas. Identificou-se, também, a partir de RT-qPCR, que os macrófagos das pacientes afetadas tinham maior expressão de IL-10 e Nlpr3. A respiração celular das pacientes afetadas também se mostrou mais ineficaz, pois a avaliação in vivo mostrou menor taxa de consumo de oxigênio (OCR) e maior acidificação extracelular (ECAR). Ao avaliar-se o padrão de depósito de gordura corporal através de tomografias computadorizadas identificou um aumento de tecido adiposo visceral na maioria das 6 pacientes afetadas avaliadas. Apesar disso, não havia sinais de complicações macro vasculares coronarianas, pois todas apresentavam o escore de cálcio coronariano normal.

4.2 – ARTIGO 1 – "DELTA-LIKE 1 HOMOLOG GENETICS AND ITS EMERGING ROLE IN HUMAN PUBERTY"

Carlos Eduardo Seraphim, Jesús Argente, Ana Claudia Latronico

Reviews

Delta-like 1 homolog genetics and its emerging role in human puberty

Carlos Eduardo Seraphim[1], Jesús Argente[2,3,4,5,6,7] and
Ana Claudia Latronico[1]

Abstract

The existence of a direct relation between weight gain and the age of pubertal onset has been demonstrated in humans. Delta-like 1 homolog (*DLK1*) acts as an adipogenesis gate-keeper by preventing adipocyte differentiation. This trans-membrane protein is a well-known noncanonical ligand of Notch signaling involved in cell state and fate. *DLK1* is encoded by an imprinted gene (paternally expressed) located on the long arm of chromosome 14 (14q32.2) that is expressed in several hypothalamic nuclei and in kisspeptin cell lines. Notably, the *DLK1* locus was associated with the age of menarche in large genome-wide association studies. More recently, several *DLK1* loss-of-function mutations were identified in children with familial central precocious puberty and in women who presented very early menarche (<9 years). Interestingly, these women had an adverse metabolic profile that was similar to that observed in *Dlk1*-null mice. *DLK1* represents a long-sought link between reproduction and metabolism.

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Introduction

Central precocious puberty (CPP) is caused by premature activation of the hypothalamic pulsatile production of gonadotropin-releasing hormone (GnRH) [1–3]. The physiological mechanism of puberty onset is partially understood but is believed to involve a shift in the balance of inhibitory, permissive, and excitatory hypothalamic factors on the GnRH neurons [2]. Several factors have a role in pubertal development, including metabolic, environmental, and genetic aspects [1,4].

The influence of genetic background on pubertal development has been historically accepted because of the observation of a similar age of menarche in mothers and daughters and in different ethnic groups [5,6]. Studies evaluating monozygotic twins showed a higher correlation of pubertal onset than those evaluating dizygotic twins [7]. Despite the fact that genetics alone responds for 60–80% of pubertal timing, the genes implicated with CPP were only recently unveiled [8,9]. The increased availability of sequencing techniques led to the identification of the first gene related to CPP, the kisspeptin receptor gene (*KISS1R or GPR54*) [10]. This finding was soon followed by the discovery of an activating mutation of the kisspeptin gene (*KISS1*) which increased intracellular signaling and was thus an enhancing factor to pubertal signaling [10,11]. Genetic studies entered the era of next-generation sequencing, which allowed the advancement from single-gene sequencing strategies to exome and genome sequencing of familial cases. In 2013, Abreu et al. [12] described loss-of-function mutations in the makorin RING-finger 3 gene. This gene represents the most common known cause of familial monogenic CPP to date [13]. It is a highly conserved imprinted gene related to ubiquitin ligases and gene transcription activity [14]. In 2017, Dauber et al. [15] described a novel gene associated with familial CPP, the delta-like 1 homolog (*DLK1*) — also known as preadipocyte factor 1 or

fetal antigen 1 — using a very innovative methodology characterized by the combination of linkage analysis followed by whole-genome sequencing analysis. This gene was of particular interest because it was known to be located at chromosome 14q32.2, a region noteworthy for being within a cluster of imprinted genes that are relevant for Temple syndrome, a very rare condition characterized by CPP (∼90%) and multiple neuro-cognitive and physical clinical features [16,17]. In addition, genome-wide association studies and longitudinal analysis had already implicated *DLK1* as one of the three imprinted loci associated with the age of menarche [18].

Notch signaling and *DLK1* functions

DLK1 is a noncanonical ligand of notch signaling, which is a highly preserved signaling process very simple in nature but very dynamic in its functions, leading to myriad of functions that involve cell state and fate [19]. Its signaling is highly regulated by both canonical and noncanonical ligands. In mammals, four Notch receptors (NOTCH1−4) and five activating canonical ligands (JAGGED1, JAGGED2, DLL1, DLL3, and DLL4) have been described [20]. Canonical ligands have a similar structure, composed of a N-terminal domain, followed by a Delta−Serrate−Lag1 motif and multiple epidermal growth factor−like (EGF-like) repetitions, and bind to NOTCH receptors with a pulling force enough to expose the negative regulatory region of the NOTCH receptor, which is processed sequentially by metalloproteinases of the a disintegrin and metalloproteinase (ADAM)/tumor necrosis factor-αα-converting enzyme (TACE) family and then by γ-secretases, leading to intracellular transcription activation [21]. *DLK1* is synthesized as a transmembrane protein that lacks the Delta−Serrate−Lag1 motif (i.e. a noncanonical ligand) and has six EGF-like repeats at the extracellular domain [22,23]. It has a short intracellular tail and a juxtamembrane region with a tumor necrosis factor-α-converting enzyme (TACE) (ADAM17)-mediated cleavage site; cleavage of this region generates a soluble portion of the *DLK1* of 50 kDa [24].

DLK1 interacts with NOTCH1 and inhibits its activation by the canonical ligands through competitive binding (Figure 1c) [18]. The EGF-like 4, 5, and 6 seem to be the most important inhibitory regions to the NOTCH1 receptor [25]. Interestingly, ubiquitination participates in this signaling, and the absence of certain E3 ubiquitin ligases prevents ligand endocytosis and signaling [22]. It is unclear whether this is relevant in puberty initiation, but it is noteworthy that the makorin RING-finger 3 gene has ubiquitin activity [23,24].

DLK1 gene and its complex regulation

DLK1 is an imprinted gene expressed only in the allele inherited from the father. It has a total size of

11.33 kb and is composed of 5 exons of which the 5th is the largest. Its transcript length is 4657 bps in humans, which corresponds to 383 residues (ENST00000341267.9) (Figure 1b). *DLK1* is located in a region called the *DLK1−DIO3* (thyroxine deiodinase type III) locus, on chromosome 14 in humans and on distal chromosome 12 in mice [30]. The region comprises three paternally expressed genes: *DLK1*, *RTL1* (a retrotransposon-like gene that participates in placental development), and *DIO3*. In contrast, the maternal allele does not express these three genes, while it does express the noncoding *GTL2/MEG3* gene and several microRNAs and small nucleolar RNAs [30]. The locus is regulated by four paternally methylated (or in cis — in the same allele) regulatory regions called differentially methylated regions (DMRs): the DLK1-DMR, the intergenic DMR (IG-DMR), the MEG-DMR, and the placenta-specific DLK1-DMR0, whose function in humans is unclear (Figure 1a) [31].

DLK1 expression

The hypothalamus is a key locus for neuronal networks intimately involved in the neuroendocrine control of puberty, harboring GnRH and kisspeptin neurons. *DLK1* is widely expressed in the embryonic tissue. Its expression is ubiquitous in embryonic development and higher in the placenta, liver, adipose tissue, skeletal muscle, lung, vertebrae, pituitary, and adrenal gland [26]. The high expression of *DLK1* in the fetus appears to induce maternal metabolic adaptations that benefit nutrient delivery and leads to rapid starvation, as shown in murine models by Cleaton et al. [27]. In the adult phase, *DLK1* expression is lower and seems to be more eminent in the pituitary, adrenal gland, pancreas, monoaminergic neurons in the central nervous system, testes, prostate, and ovaries [26,28]. *DLK1* expression decreases substantially in all tissues except endocrine glands during the postnatal period. Villanueva et al. [23] first characterized *Dlk1* expression in the mouse hypothalamus. Immunohistochemistry showed neuronal *DLK1* expression in the suprachiasmatic, supraoptic, paraventricular, arcuate, dorsomedial, and lateral hypothalamic nuclei. More recently, *DLK1* expression was detected in mouse medio basal hypothalamus and in two kisspeptin neuron-derived cell lines [15]. These data provide further supportive evidence that *DLK1* may have a role in regulating pubertal timing, as well as prenatal and early postnatal growth of the conceptus [24].

Loss-of-function mutations of *DLK1*

In 2017, linkage analysis followed by whole-genome sequencing was performed in a Brazilian multigenerational family with five female members affected by nonsyndromic CPP [15]. A very complex defect of *DLK1* (∼14-kb deletion and 269-bp duplication) was identified in this family [15]. This deletion included the 5′ untranslated region and the first exon of *DLK1*,

Figure 1
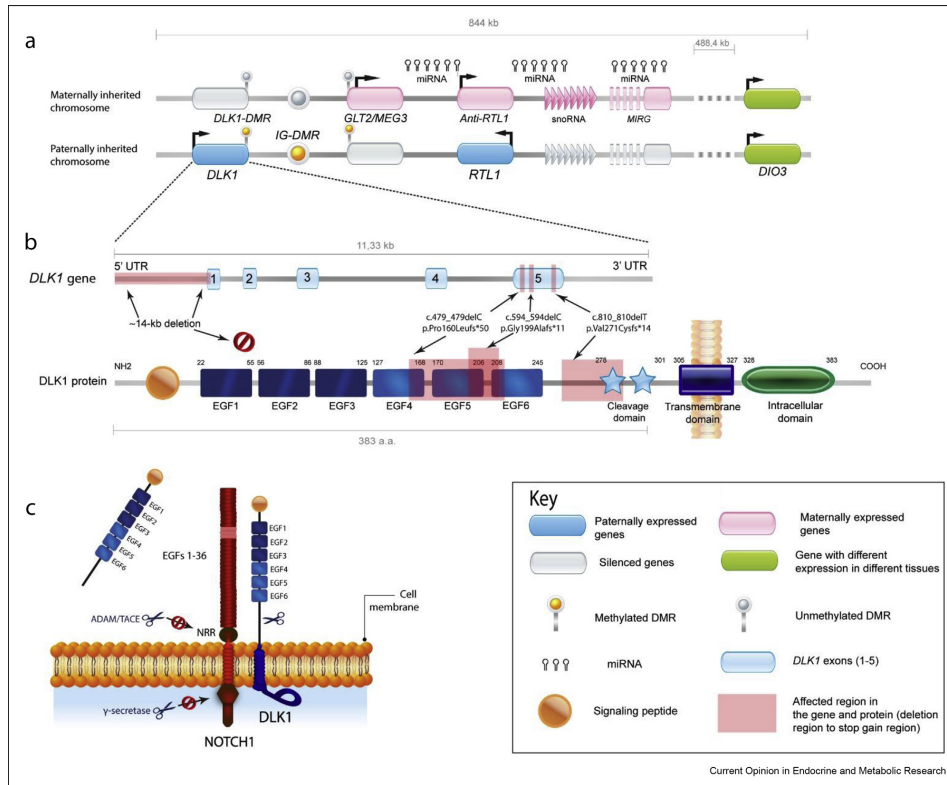
***DLK1* gene expression and protein and NOTCH1 interaction. (a)** – The *DLK-DIO3* locus is represented in the alleles inherited from both parents. While the paternally inherited chromosome expresses *DLK1* and *RTL1* genes, the maternally inherited chromosome expresses the *GLT2/MEG3* gene, which is a noncoding gene that has an important role as a tumor suppressor. Several miRNAs and snoRNAs are also expressed in the maternally inherited chromosome. The last gene in the locus, *DIO3*, is differentially expressed according to each tissue, both from maternal and paternal chromosomes, through poorly known mechanisms. The DLK1-DMR is located between intron 4 and exon 5 of the *DLK1*. However, the most important DMR seems to be the IG-DMR, which is methylated during spermatogenesis in the paternal allele and unmethylated in the maternal allele [30]. The methylated IG-DMR apparently coordinates the pattern of methylation/expression of the other genes: the methylation and expression of *DLK1*, expression of *RTL1* and *DIO3* and the methylation and inactivation of the *MEG3* (which is active in the maternal allele). **(b)** – *DLK1* is composed of 5 exons. This figure depicts both the *DLK1* gene and the corresponding protein with its domains. The four different mutations reported are also represented. The 14 kb deletion affects the translational start site, leading to loss of translation of the *DLK1* gene. The other three frameshift mutations are represented. The red boxes in the protein represent the deletion region on its left side and the stop gain region on its right side. Both p.Pro160Leufs*50 and p.Gly199Alafs*11 affect the region of EGFs 4, 5, and 6, which are most important in interacting with NOTCH receptors. The p.Val271Cysfs*14 mutation is located after the EGF6 but generates a premature stop codon in the cleavage domain of the protein. **(c)** – This figure represents the interaction of the DLK1 protein (both in its transmembrane and its soluble forms) and the NOTCH1 receptor. The interaction of *DLK1* with NOTCH1 receptor inhibits notch signaling through competitive binding. This interaction does not expose the NRR region for cleavage by the ADAM/TACE; therefore, the intracellular NOTCH domain is not cleaved by the γ-secretase. Evidence shows that the NOTCH1's EGFs 11, 12, and 13 (depicted in lighter red) interact with DLK1's EGFs 4, 5, and 6 (depicted in lighter blue). kb = kilobase; miRNA = microRNA; snoRNA = small nucleolar RNA; *DLK1* = delta-like 1 homolog; *GLT2/MEG3* = gene trap locus 2/maternally expressed gene 3; *RTL-1* = retrotransposon-like 1; *DIO3* = Iodothyronine Deiodinase 3; *DLK1-DMR* = differentially methylated region of the *DLK1* gene; *IG-DMR* = intergenic differentially methylated region; *MIRG* = miRNA containing gene; UTR = untranslated region; EGF-like = epidermal growth factor−like; a.a. = amino acids; NRR = negative regulatory region.

including the translational start site. Only family members who inherited the defect from their father have CPP, consistent with the known imprinting of *DLK1*. The affected patients did not demonstrate additional features of an imprinted disorder, such as Temple syndrome, except for increased fat mass detected by bioimpedance. This complex genomic alteration was the first evidence of *DLK1* deficiency causing premature sexual development in humans. In addition, the prevalent clinical manifestation of CPP among the complex phenotype features of Temple syndrome was also attributed to *DLK1* loss of function [25].

More recently, 60 female patients with a diagnosis of idiopathic CPP or history of precocious menarche were screened for potential *DLK1* mutations [26]. Three frameshift mutations of *DLK1* (p.Gly199Alafs*11, p.Val271Cysfs*14, and p.Pro160Leufs*50) in five women from three unrelated families with CPP were identified (Table 1). Segregation analysis was consistent with the maternal imprinting of *DLK1*. Interestingly, serum *DLK1* levels were undetectable in these patients and a metabolic syndrome phenotype was more prevalent than in other idiopathic patients with CPP, with 60% of them having truncal overweight/obesity, 70% insulin resistance, 30% type 2 diabetes mellitus, and 50% hyperlipidemia. Metabolic abnormalities, such as overweight/obesity, early-onset glucose intolerance/type 2 diabetes mellitus, and hyperlipidemia, were more prevalent in women with the *DLK1* mutation than in the idiopathic CPP group [26]. Notably, the human metabolic

alterations were similar to the previously described *Dlk1*-null mice phenotype [27]. These animals had accelerated weight gain due a higher degree of adipose tissue production, enlarged fatty liver, and hyperlipidemia (Figure 2) [27,28]. All patients with *DLK1* loss-of-function mutations had normal birth weight, which is in agreement with animal models of *Dlk1*-null mice, which had also normal birth weight but increased proportion of adipose tissue [29]. Mice overexpressing *Dlk1* had normal birth weight as well, but they had limited accumulation of adipose tissue during postnatal life [30]. This finding suggests that *DLK1* is important for lipid

Figure 2

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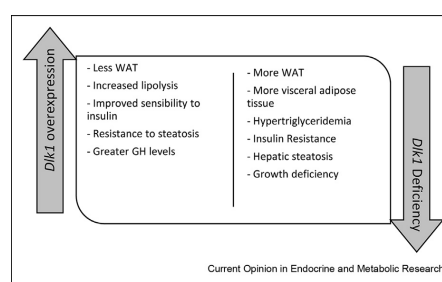
Effects of *Dlk1* deficiency and overexpression in animal models. WAT = white adipose tissue; GH = growth hormone. Data regard the phenotype of *Dlk1*-null mice and transgenic mice that overexpressed *Dlk1* [29,33].

Table 1

Pathogenic variants in *DLK1* in patients with CPP or precocious menarche.

Pathogenic variants	Affected individuals (n)	Effect on protein	Clinical features and familial history
~14-kb deletion and 269-bp duplication	5F	Loss of exon 1 and translational start region	• Four female members had CPP • Paternal grandmother had menarche 9−10 years-old. • Higher % fat mass • Obesity • 1/5 type 2 diabetes
c.594_594delC p.Gly199Alafs*11	2F	Frameshift leads to premature stop codon in EGF-like region 5	• Both had CPP • Both had PCOS, infertility, type 2 diabetes, hepatic steatosis
c.810_810delT p.Val271Cysfs*14	1M1F	Frameshift leads to premature stop codon after EGF-like region 6	• Both had CPP • Familial history of paternal relatives with CPP
c.479_479delC p.Pro160Leufs*50	2F	Frameshift leads to premature stop codon in EGF-like region 4	• One had CPP • Paternal aunt had menarche around 9 y.o. • One has glucose intolerance and hypercholesterolemia

M = male; F = female; EGF-like = epidermal growth factor−like; CPP = central precocious puberty; PCOS = polycystic ovary syndrome.
Cases were reported by Dauber et al. [15] and Gomes et al. [25].

storage during lifetime, and its loss leads to a higher accumulation of white adipose tissue (WAT).

Two sisters who carried the p.Gly199Alafs*11 mutation also exhibited polycystic ovary syndrome and infertility. This finding might be in agreement with the neuroendocrine component implicated in polycystic ovary syndrome physiopathology, as the increased GnRH pulse frequency might lead to increased luteinizing hormone (LH) production over follicle-stimulating hormone (FSH) production. Loss-of-function mutations of *DLK1* are currently considered a definitive and rare cause of familial CPP. The high prevalence of metabolic alterations in adult women who experienced CPP due to *DLK1* defects suggests that this antiadipogenic factor represents a link between reproduction and metabolism. The localization of the current pathogenic variants of *DLK1* is presented in Figure 1b.

Circulating *DLK1* levels

The soluble form of *DLK1*, with a molecular weight of 50 kDa, can be generated through TACE-mediated cleavage of its extracellular domain. Interestingly, serum *DLK1* concentrations were undetectable in girls with CPP caused by deleterious genetic defects of *DLK1*, supporting the notion that these pathogenic variants lead to complete lack of *DLK1* production in these individuals [15,26]. Similar data were observed in patients with Temple syndrome who had barely detectable levels of *DLK1* independent of their sex, age, or the molecular defect of 14q32.2 [31]. These findings suggest that this accessible biochemical measurement could be a potential screening assay for the diagnosis of isolated and syndromic familial CPP due to a rare deficiency of *DLK1*.

DLK1 and metabolism

There is a myriad of evidence correlating *DLK1* with metabolic aspects, in particular adipogenesis. It has an inhibitory effect over adipogenesis, preventing the differentiation of preadipocytes into mature adipocytes. Its expression is high in preadipocytes and completely absent in mature adipocytes; murine models have previously shown that *DLK1* affects adipogenesis by interacting with fibronectin and activating the mitogen-activated protein kinases/extracellular signal-regulated kinases (MERK/ERK) pathway to induce Sox9 expression, thus inhibiting the key transcriptional regulators of adipogenesis CEBPα (CCAAT/enhancer binding protein α) and PPARγ (peroxisomeproliferator-activated receptor γ) [32,33]. Therefore, *DLK1* overexpression leads to lower expansion of WAT, while *DLK1* deficiency increases adiposity [34]. Murine models of intrauterine restriction showed that lower *DLK1* levels led to visceral fat expansion and insulin resistance [27]. Other features observed in animal models of higher expression of

DLK1 and deficiency are summarized in Figure 2. A recent study demonstrated that *DLK1* seems to be important for beige fat biogenesis and adaptive thermogenesis, increasing the role of *DLK1* beyond WAT function [35].

An interesting study using murine models performed by Charalambous et al. [30] demonstrated that mice overexpressing *Dlk1* had reduced fat stores and reduced liver steatosis, even when fed with a high fat diet. This pointed to a role of *DLK1* in signaling against fat accumulation and toward peripheral lipid oxidation, which is metabolically beneficial [30]. Another interesting aspect is the presence of *DLK1* in orexin- and dynorphin-containing neurons, which are involved in appetite upregulation, and absence of *DLK1* in cocaine- and amphetamine-regulated transcript—containing neurons [36,37]. One could speculate that *DLK1* might be a signal of the energy oxidation status, at the same time blocking fat storage and optimizing lipid oxidation and acting on neurohormonal circuits that regulate hunger to favor energy consumption [36].

Unexpectedly, a recent study performed in 48 Danish men showed a positive correlation of *DLK1* serum levels and body mass index. *DLK1* was also positively correlated to increased central fat mass, due to increased visceral fat mass [38,39]. These findings resemble somehow data about leptin in humans, while congenital leptin deficiency leads to early-onset severe obesity, patients with acquired obesity have higher levels of leptin due to leptin resistance.

Conclusions

The association of *DLK1* loss-of-function mutations with familial CPP not only expanded the list of monogenic causes of CPP but also opened a new insight into the correlation between obesity/metabolic syndrome and pubertal development. Historically, the permissive role of adipokines such as leptin and ghrelin over puberty has been extensively described together with the increased prevalence of metabolic syndrome in patients with earlier menarche to point out the important correlation between the reproductive axis and metabolic features [40,41]. The increasing knowledge about *DLK1* deficiency is a promising pathway. On the one hand, the possible inhibition of notch signaling in permissive pubertal pathways, such as kisspeptin, could enhance comprehension about the complex mechanism of pubertal timing. On the other hand, the underestimated role of notch signaling and *DLK1* in obesity and metabolic syndrome is being unveiled and could lead to new paths to understand these relevant diseases.

Conflict of interest statement

Nothing declared.

Acknowledgements

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Papers of particular interest, published within the period of review, have been highlighted as:

    *   of special interest
    ** of outstanding interest

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4.3 – ARTIGO 2 – "GENOTYPE–PHENOTYPE CORRELATIONS IN CENTRAL PRECOCIOUS PUBERTY CAUSED BY MKRN3 MUTATIONS"

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Clinical Research Article

Genotype–Phenotype Correlations in Central Precocious Puberty Caused by *MKRN3* Mutations

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Abstract

Context: Loss-of-function mutations of makorin RING finger protein 3 (MKRN3) are the most common monogenic cause of familial central precocious puberty (CPP).

Objective: To describe the clinical and hormonal features of a large cohort of patients with CPP due to *MKRN3* mutations and compare the characteristics of different types of genetic defects.

Methods: Multiethnic cohort of 716 patients with familial or idiopathic CPP screened for *MKRN3* mutations using Sanger sequencing. A group of 156 Brazilian girls with idiopathic CPP (ICPP) was used as control group.

Results: Seventy-one patients (45 girls and 26 boys from 36 families) had 18 different loss-of-function *MKRN3* mutations. Eight mutations were classified as severe (70% of patients). Among the 71 patients, first pubertal signs occurred at 6.2 ± 1.2 years in girls and 7.1 ± 1.5 years in boys. Girls with *MKRN3* mutations had a shorter delay between puberty onset and first evaluation and higher follicle-stimulating hormone levels than ICPP. Patients with severe *MKRN3* mutations had a greater bone age advancement than patients with missense mutations (2.3 ± 1.6 vs 1.6 ± 1.4 years, $P = .048$), and had higher basal luteinizing hormone levels (2.2 ± 1.8 vs 1.1 ± 1.1 UI/L, $P = .018$) at the time of presentation. Computational protein modeling revealed that 60% of the missense mutations were predicted to cause protein destabilization.

Conclusion: Inherited premature activation of the reproductive axis caused by loss-of-function mutations of *MKRN3* is clinically indistinct from ICPP. However, the type of genetic defect may affect bone age maturation and gonadotropin levels.

Key Words: precocious puberty, *MKRN3*, genetic of puberty, *MKRN3* phenotype

Makorin RING finger 3 (*MKRN3)* is an intronless gene located inside a region containing an imprinted gene cluster at chromosome 15q11-q13. It undergoes maternal imprinting; therefore, only the paternal allele is expressed, while the maternal allele is silenced through methylation of CpG islands (1, 2). MKRN3 function is associated with gene transcription and E3 ubiquitin ligase activity (3, 4).

MKRN3 inactivating mutations were first described in 15 patients of both sexes (8 girls and 7 boys) from 5 different families with central precocious puberty (CPP) using whole-exome sequencing analysis in 2013 (5). Different types of mutations (frameshift, stop gain, missense) affecting the MKRN3 protein or the gene promoter region have been subsequently described after this work (4) and *MKRN3* defects have been recognized as the most common monogenic cause of familial CPP. Notably, patients with CPP due to *MKRN3* mutations have typical clinical and hormonal features of premature activation of the reproductive axis, including early pubertal signs, such

as breast and pubic hair development, accelerated linear growth, advanced bone age, and elevated basal and/or gonadotropin-releasing hormone (GnRH)–stimulated luteinizing hormone (LH) levels. Macedo et al. (6) described that the age of pubertal onset ranged from 3.0 to 6.4 years (mean 5.3 years and median 6.0 years) in a small group of Brazilian girls with *MKRN3* mutations. Except for the significantly higher levels of basal follicle-stimulating hormone (FSH) in patients with *MKRN3* mutations than in those without *MKRN3* mutations, no other clinical or hormonal differences were identified between these 2 groups.

A recent systematic review that gathered cohorts from 22 different studies (>800 patients analyzed) revealed a high prevalence of loss-of-function mutations in *MKRN3* in patients with familial CPP from different parts of the world, with higher frequency in Occidental countries (4). In the current study, we describe detailed phenotypic characteristics of the largest multiethnic cohort of patients with CPP harboring loss-of-function mutations of *MKRN3*

evaluated by one medical center. Furthermore, we compare clinical data between patients with different types of mutations and perform in silico analysis of the stability of the protein for missense variants.

Patients and Methods

Study design and participants

Clinical data were extracted from a large cohort of patients with familial or idiopathic CPP (716 cases, 64% females) referenced to a Brazilian Medical Center for molecular analysis (Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil). All index cases had CPP, defined by the development of progressive pubertal signs before 8 years in girls and before 9 years in boys (7). All index patients had CPP confirmed either by pubertal basal (>0.2 UI/L) or GnRH-stimulated LH levels (>5 UI/L by electrochemiluminescence: ECLIA), and they had organic causes ruled out by magnetic resonance imaging of the central nervous system (7, 8).

In order to identify clinical or laboratory predictors of *MKRN3* mutations, we assessed data from another cohort of 156 girls with idiopathic CPP followed in the Brazilian Center. All of them were negative for *MKRN3* mutations, as well as negative for other established monogenic causes of CPP. Their data were compared with data from the group of individuals with *MKRN3* mutations. Because of the low number of boys with CPP (idiopathic and associated with MKRN3 mutations) the same comparison could not be performed.

Clinical and laboratory data collection

The Endocrinology Division of the Hospital das Clínicas in Sao Paulo, Brazil, is a tertiary center; patients were referred from other Brazilian states to investigate genetic causes of CPP. Additionally, we received DNA samples and clinical data from other countries through international collaborations and from a multiethnic cohort encompassing 716 subjects, including 517 index patients and their close relatives. The largest group of subjects were Brazilian (371), whereas the remaining 345 were from other countries (the largest cohort being 228 Spanish cases). Sanger sequencing for *MKRN3* was performed in all index cases (517 of the 716 subjects) and 71 individuals had *MKRN3* pathogenic variants (36 index cases and 35 relatives). Medical records were systematically reviewed from all confirmed *MKRN3* mutated patients, and all available data were collected, including follow-up and treatment information. Relevant clinical data was gathered as follows: (1) age at first pubertal signs (breast buds in girls and testicular enlargement in boys); (2) age at pubarche; (3) age at first evaluation – chronological age (CA); (4) bone age at first evaluation (evaluated according to Greulich and Pyle method); (5) bone age advancement (bone age advance = bone age – chronological age); (6) height (standard deviation score [SDS]); (7) weight; (8) body mass index and body mass index-SDS (9). The laboratory data included basal and GnRH-stimulated LH and FSH levels, measured either by immunochemiluminometric or ECLIA assays; and basal estradiol or testosterone levels, measured by immunoassays or liquid chromatography-tandem mass spectrometry. Pubertal LH basal values were considered higher than 0.2 UI/L (ECLIA) and the GnRH-stimulated LH levels cutoff was 5 UI/L using ECLIA (7). Whenever available, data regarding follow-up, treatment, and final height were also collected and analyzed in comparison with the predicted target height.

Genetic and molecular analysis

MKRN3 sequencing analysis

Genomic DNA was extracted from peripheral blood leukocytes. We analyzed the coding region of *MKRN3* for allelic variants by performing PCR amplification followed by sequencing of the products with the use of the conventional Sanger method, as previously described (5). The local ethics committees (Comissão de Ética para Análise de Projetos de Pesquisa—CAPPesq) approved this study and all individuals and/or their legal guardians gave their written informed consent. *DLK1* mutations were excluded in all patients described in this study. A subset of patients with CPP had *KISS1* and *KISS1R* mutations also excluded (5).

All pathogenic variants were evaluated according to the American College of Medical Genetics (ACMG) criteria (10). In order to establish genotype–phenotype correlations, pathogenic variants were classified and grouped either as severe (frameshift, stop gain, and promoter region mutations) or missense mutations. The rationale behind this categorization was that in the first group the mutations led to truncated and possibly more dysfunctional proteins, which could lead to a more pronounced phenotype.

MKRN3 protein stability analysis

Computational protein modelling analysis was performed using Yasara software (Vienna, Austria) with FoldX toolsuite, which provided an analysis of the effect of mutations on the stability, folding and dynamics of proteins (11). This type of analysis first calculates the free energy of unfolding (ΔG) of a target protein. Subsequently, it calculates the energy difference between the wild type and a variant of the protein (ΔΔG) (11).

Statistical analysis

Data were described by simple descriptive statistics or frequencies and percentages. Whenever appropriate, bootstrapping analysis of smaller number of observations was performed to reduce possible bias due to sampling. Data are presented as mean and standard deviation unless otherwise stated. Comparisons between the different groups of mutations and between the genetic and idiopathic cases included Student's t-test or Wilcoxon signed-rank test for numerical continuous variables as appropriate. Categorical variables were compared between groups using the chi-squared test or Fisher's exact test as appropriate. Paired t-tests were conducted in the subgroup of treated patients. Statistical analysis was performed in R Studio (version 1.2.1335) and $P < .05$ was considered statistically significant.

Results

MKRN3 pathogenic variants

Among 716 cases screened for *MKRN3* sequence variants, we identified 71 patients (45 girls and 26 boys) from 36 unrelated families who had pathogenic variants according to ACMG criteria. This cohort comprised 9 different ethnic backgrounds: 40 Brazilian, 9 American, 8 Spanish, 5 Argentinean, 4 Belgian, 2 Israelis, 1 Australian, 1 Norwegian, and 1 Turkish (Fig. 1). Thirty-seven patients were newly reported cases, and the remaining 34 patients were previously described and included for statistical analysis (4–6, 12). Twenty-one families were available for segregation analysis.

Eighteen different *MKRN3* mutations were identified. Eight of them were classified as severe: 6 different frameshift variants, 1 stop gain variant, and 1 promoter region deletion. The promoter region deletion was previously reported and leads to the loss of a transcription factor binding site and was thus considered severe as well (13). The severe mutations of *MKRN3* occurred in the great majority of the cohort with CPP: 53 out of 71 patients (75%); or 26 out of 36 index cases (72%). The remaining 10 mutations were missense pathogenic variants (18 out of 71 patients—25%). All identified *MKRN3* mutations are represented in Fig. 2.

It is worth noting that the most prevalent *MKRN3* mutations, occurring in 34 (46%) patients, were indel variants affecting a poly-C region in cDNA positions 475 to 481 (7 cytosines), which caused frameshift mutations and premature stop codons. These variants have been previously named in a few different ways, generally in the amino acid positions 161 and 162. Here, we adopted a single nomenclature for this hotspot region (p.Pro161Argfs*), because Sanger sequencing reading might differ due to the poly-C region.

Missense variants pathogenicity assessment

According to ACMG criteria, 10 missense *MKRN3* variants were categorized either as pathogenic or likely pathogenic (Table 1). In addition, according to Foldx and Yasara software analysis, 6 of them were identified as destabilizing or highly destabilizing, while 4 were identified as neutral or stabilizing. However, missense variants identified as stabilizing or neutral were located in critical regions of the protein structure. The 2 most common regions affected by
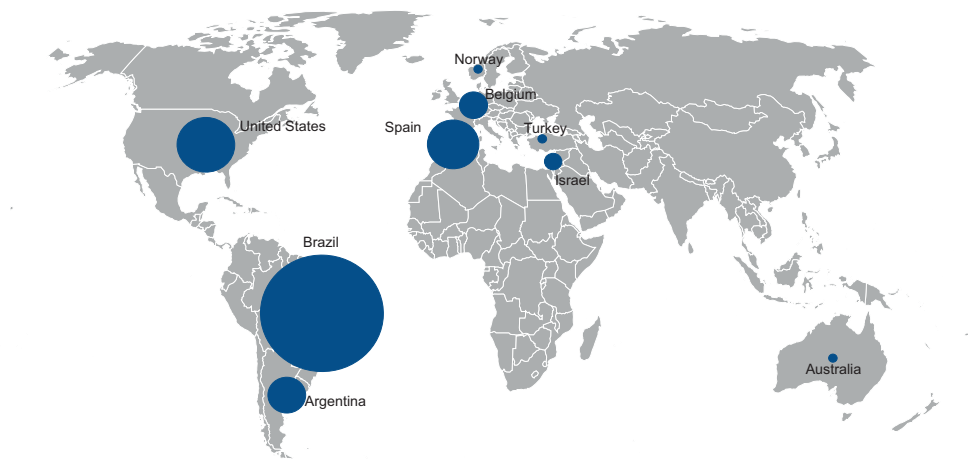
Figure 1. Geographic distribution of 71 patients with CPP due to MKRN3 mutations. The larger circles represent higher numbers of cases: 40 in Brazil, 9 in USA, 8 in Spain, 5 in Argentina, 4 in Belgium, 2 in Israel, 1 in Norway, 1 in Australia, and 1 in Turkey.
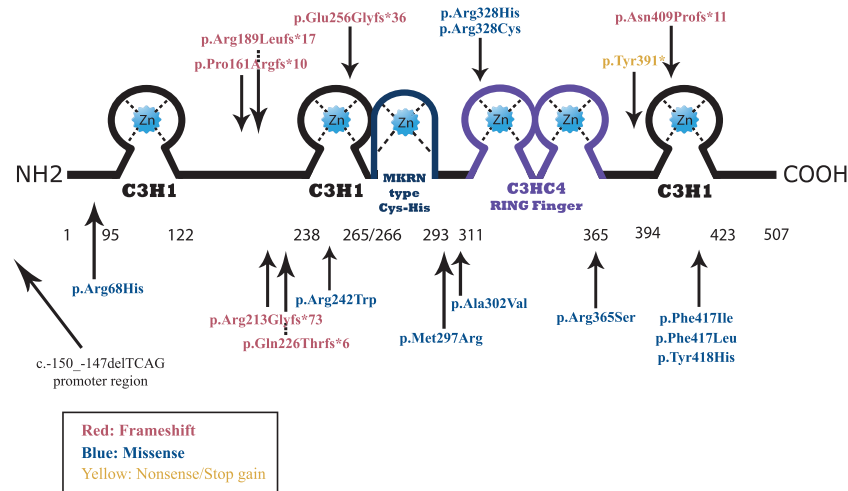
Figure 2. Schematic representation of MKRN3 protein and location of severe (red and yellow) and missense (blue) variants. The MKRN3 protein is composed of 3 C3H1 zinc fingers, a MKRN type Cys-His region, and a C3HC4 RING finger. The arrows point to the corresponding region of the mutation in the protein. The promoter region deletion is represented in this image without a corresponding region in the protein because it leads to loss of transcription due to the loss of a DREAM binding site (13).

missense variants were the third C3H1 ring finger and the C3HC4 ring finger of the protein (Table 2).

Clinical and laboratory features

Among the cohort with *MKRN3* mutations (71 total cases), first pubertal signs occurred at 6.2 ± 1.2 years in girls and 7.1 ± 1.5 years in boys, and they had a bone age advancement of 2.0 ± 1.6 and 1.8 ± 1.3 years, respectively. Hormonal data showed basal LH levels of 1.9 ± 1.8 IU/L in girls and 1.6 ± 1.2 IU/L in boys. More detailed clinical and hormonal data is provided in Table 3.

Genotype–phenotype correlations

The 53 patients who harbored severe *MKRN3* mutations had a greater bone age advancement than those with missense mutations (2.3 ± 1.6 vs 1.6 ± 1.4 years, respectively, *P* = .048), and also had higher basal LH levels (2.2 ± 1.8 vs 1.1 ± 1.1 UI/L, *P* = .018). Other clinical and laboratory features were not significantly different between the 2 groups (severe vs missense) and are depicted in Table 4.

Phenotype comparison between girls with CPP with and without *MKRN3* mutations

The 45 girls with CPP caused by *MKRN3* loss-of-function mutations were compared with a cohort of 156 Brazilian girls with idiopathic CPP. Girls with *MKRN3* mutations had a shorter delay between puberty onset and first evaluation (0.8 ± 0.8 vs 2.4 ± 2.1 years, respectively, *P* < .001), which occurred at a younger age (7.2 ± 1.1 years vs 8.4 ± 2.0 years; *P* < .001). Among the *MKRN3* subjects, those who had a positive family history of precocious puberty sought medical assistance earlier than those *MKRN3* patients without family history (0.42 ± 0.43 vs 1.1 ± 1 years; *P* < .01). Interestingly, though, even when we compared only the *MKRN3* patients without family history of CPP to the cohort of idiopathic CPP the interval remained shorter in the *MKRN3* group (1.1 ± 1.0 vs 2.4 ± 2.0; *P* < .001).

Furthermore, girls with CPP caused by *MKRN3* mutations presented with less pronounced height SDS increase at first evaluation (1.2 ± 1.2 vs 1.7 ± 1.1; *P* = .04). They also presented with higher basal FSH levels than girls with idiopathic CPP (4.9 ± 2.3 IU/L vs 3.8 ± 2.7IU/L; *P* = .03). However, no cut-off level was established, as the values overlapped. All data are shown in Table 5.

Follow-up and response to GnRH analogue treatment

Among the 71 patients with CPP due to MKRN3 mutations, final height was available for 24 patients treated with GnRH depot analogues and for 8 untreated adults. The untreated adults were diagnosed lately (adult phase) through family screening. Treated patients had a mean time

Table 1. MKRN3 missense variants identified in patients with CPP and pathogenicity assessment through ACMG criteria

Position	cDNA	Protein	rs	Population data (AF)		ACMG criteria	
				gnomAD	ABraOM	Detailed staging	Classification
15:23812024	c.1095G>T	p.Arg365Ser	rs879255240	0.00003976	Not reported	PS4, PM2, PP1, PP3, PP4, PP5	Pathogenic
15:23812178	c.1249T>A	p.Phe417Ile	NA	Not reported	Not reported	PS4, PM2, PP1, PP3, PP4, PP5	Pathogenic
15:23811132	c.203G>A	p.Arg68His	rs149274884	0.00001201	Not reported	PM2, PP1, PP2, PP4, PP5, BP4	Likely pathogenic
15:23811653	c.724C>T	p.Arg242Trp	rs1277371835	0.00001193	Not reported	PM2, PP1, PP2, PP4, BP4	Likely pathogenic
15:23811834	c.905C>T	p.Ala302Val	NA	Not reported	Not reported	PS4, PM2, PP1, PP3, PP4	Likely pathogenic
15:23811912	c.983G>A	p.Arg328His	rs1355723562	0.00006371	Not reported	PS4, PM2, PP1, PP3, PP4	Likely pathogenic
15:23811911	c.982C>T	p.Arg328Cys	rs1355723562	0.00006371	Not reported	PS4, PM2, PP1, PP3, PP4	Likely pathogenic
15:23812178	c.1249T>C	p.Phe417Leu	rs745560329	0.00003976	Not reported	PM2, PM5, PP1, PP2, PP4, BP4	Likely pathogenic
15:23812181	c.1252T>C	p.Tyr418His	rs1470111765	0.00003976	Not reported	PM2, PP2, PP1, PP2, PP3, PP4	Likely pathogenic
15:23811819	c.890T>C	p.Met297Arg	rs147605349	0.00001193	Not reported	PM2, PP1, PP2, PP3, PP4	Likely pathogenic

Abbreviations: AF, allele frequency; ACMG, American College of Medical Genetics and Genomics; gnomAD, Genome Aggregation Database; ABraOM, Online Archive of Brazilian Mutations; ACMG criteria are given according to reference number 10; PS4, pathogenic strong 4; PM2, pathogenic moderate 2; PM5, pathogenic moderate 5; PP1, pathogenic supporting 1; PP2, pathogenic supporting 2; PP3, pathogenic supporting 3; PP4, pathogenic supporting 4; PP5, pathogenic supporting 5; BP4, benign supporting 4.

of treatment of 2.9 ± 0.9 years, and mean final height SDS was −0.46 ± 0.61, which was indistinguishable from their mean target height of −0.69 ± 0.86 (*P* = .37).

Discussion

MKRN3 loss-of-function mutations represent the most common genetic cause of familial CPP (8). They have been described in children from multiple countries and may account for a substantial proportion of inherited CPP cases (33-46%) (12, 14). In the present study, we investigated the genotype and phenotype features of a multiethnic cohort (Latin American, North American, European, Israeli, and Turkish subjects) with deleterious defects of *MKRN3*. The cohort was composed of 71 patients (from 36 unrelated families) with CPP caused by 18 *MKRN3* inactivating mutations. Both female and male patients carrying *MKRN3* mutations exhibited classical clinical and biochemical features of premature reactivation of the reproductive axis. Girls started pubertal development at a mean age of 6.2 ± 1.2 years, whereas in boys it was at 7.1 ± 1.5 years. Higher levels of basal FSH and an earlier age at diagnosis were identified in female patients with CPP associated with *MKRN3* when compared with an idiopathic CPP group from Brazil, in agreement with the previous study of Macedo et al. (6). The higher FSH levels might be attributed to different frequencies of pulsatile GnRH release and its impact on gonadotropin secretion. While low GnRH pulse frequencies stimulate FSH secretion preferably, higher GnRH pulse frequencies favor LH secretion (15). Therefore, it is reasonable to hypothesize that *MKRN3* loss of function could lead to a lower frequency pattern of GnRH pulsatility, which in turn could lead to higher FSH secretion.

The shorter interval between initial manifestations and diagnosis of CPP in patients with *MKRN3* mutations was probably related to the fact that 51% of them had a familial history of precocious puberty, increasing the awareness of parents and doctors for premature sexual development in a second case in the same family.

The determination of age at pubertal onset in boys is usually a challenge, because testicular enlargement (first male pubertal sign) is not as obvious as thelarche and menarche in girls. In addition, male patients usually remember only late events of puberty, such as the age at initiation of full facial shaving and the age at voice change. Bessa et al. (12) demonstrated a high frequency of *MKRN3* mutations in a series of 20 boys with CPP, previously classified as idiopathic, suggesting the importance of genetic analysis in this group. Notably, boys with CPP due to *MKRN3* mutations had puberty initiation at a borderline age according to several studies (7.9-8.5 years) (12, 16, 17). This fact can

Table 2. Protein stability analysis of MKRN3 with missense variants

Mutation (missense)	ΔΔG	Protein stability	Location within the protein
p.Arg68His	0.915	Slightly destabilizing	Before C3H1 zinc finger 1
p.Arg242Trp	−1.090	Stabilizing	Second C3H1 zinc finger
p.Met297Arg	−0.240	Neutral	Between MRKN type Cys-His domain and C3HC4 ring finger
p.Ala302Val	1.000	Destabilizing	Between MRKN type Cys-His domain and C3HC4 ring finger
p.Arg328His	1.409	Destabilizing	C3HC4 ring finger
p.Arg328Cys	1.661	Destabilizing	C3HC4 ring finger
p.Arg365Ser	1.617	Destabilizing	C3HC4 ring finger
p.Phe417Ile	−1.010	Stabilizing	Third C3H1 zinc finger
p.Phe417Leu	−1.392	Stabilizing	Third C3H1 zinc finger
p.Tyr418His	2.882	Highly destabilizing	Third C3H1 zinc finger

ΔΔG is the energy difference between the wild type and a variant of MKRN3. This analysis can provide a classification of the mutation into 7 categories: (1) highly stabilizing (ΔΔG < −1.84 kcal/mol); (2) stabilizing (−1.84 kcal/mol ≤ ΔΔG < −0.92 kcal/mol); (3) slightly stabilizing (−0.92 kcal/mol ≤ ΔΔG < −0.46 kcal/mol); (4) neutral (−0.46 kcal/mol < ΔΔG ≤ +0.46 kcal/mol); (5) slightly destabilizing (+0.46 kcal/mol < ΔΔG ≤ +0.92 kcal/mol); (6) destabilizing (+0.92 kcal/mol < ΔΔG ≤ +1.84 kcal/mol); and (7) highly destabilizing (ΔΔG > +1.84 kcal/mol) [11].

Table 3. Clinical and hormonal features of 71 patients with CPP caused by *MKRN3* mutations

	Girls (n = 45) Mean ± SD	Boys (n = 26) Mean ± SD
First pubertal signs (years)	6.22 ± 1.19	7.13 ± 1.46
Pubarche (years)	6.96 ± 1.14	7.3 ± 1.58
At first evaluation		
Chronological age (years)	7.13 ± 1.05	8.00 ± 1.53
BA (years)	9.17 ± 1.98	9.51 ± 2.56
BAA (years)	2.04 ± 1.57	1.81 ± 1.30
Height SDS	1.25 ± 1.06	0.43 ± 1.10
BMI SDS	0.92 ± 0.99	1.50 ± 1.26
Basal LH (IU/L)	1.86 ± 1.78	1.59 ± 1.22
Basal FSH (IU/L)	4.94 ± 2.33	2.64 ± 1.87
Estradiol (ng/dL)	30.0 ± 20.9	
Testosterone (ng/dL)	.	186 ± 185
LH peak (IU/L)	20.16 ± 14.93	10.87 ± 4.98
FSH peak (IU/L)	16.94 ± 4.86	
Treatment duration (years)	2.89 ± 0.91 (n = 20)	2.72 ± 0.57 (n = 5)
Age at menarche in treated subjects (years)	11.3 ± 1.2	
Age at menarche in untreated subjects (years)	8.2 ± 1.0	
Final height SDS in treated patients	−0.46 ± 0.61[a] (n = 16)	−0.9 ± 1.51 (n = 3)
TH SDS for treated patients	−0.69 ± 0.86[a]	−0.1 ± 0.88 (n = 3)

Data are shown as mean ± standard deviation.

Abbreviations: BA, bone age; BAA, bone age advance; SDS, standard deviation score; LH, luteinizing hormone; FSH, follicle stimulating hormone; TH, target height; NA, not applicable.

[a]Final height in treated girls did not differ from predicted target height (*P* = .37).

compromise the precise identification of puberty, leading to an underestimate of the incidence of CPP in the male group. Indeed, in the current study, the clinical data of boys with MKRN3 CPP was also more scarce than for girls, mainly because 87.5% of all male subjects were diagnosed through familial screening; only 5 boys were index cases (out of 36 index cases). Some of the rest of them were underdiagnosed in childhood and the CPP history was only recognized retrospectively in adult life, while others were siblings of index patients and had an earlier diagnosis that might have been undetected otherwise. Therefore, we believe that male CPP caused by *MKRN3* mutations can be clinically subtle.

To date, all patients with *MKRN3* loss-of-function mutations have a paternal origin when familial segregation analysis was possible. A documented de novo *MKRN3* mutation has not been described to date, indicating that true sporadic cases are very uncommon. In fact, a history of premature sexual development on the paternal side can

Table 4. Phenotype comparison between severe and missense variants of *MKRN3*

	Severe pathogenic[a]	Missense pathogenic[b]	P
Number of patients (%) number of families	53 (75%) 26	18 (25%) 10	NA
Male/Female	18/35	8/10	NA
First pubertal signs (years)	6.2 ± 1.3	6.8 ± 1.2	.23
Pubarche (years)	6.8 ± 1.1	7.6 ± 1.3	.19
First evaluation			
CA (years)	7.3 ± 1.1	7.5 ± 1.5	.62
BAA (years)	2.3 ± 1.6	1.6 ± 1.4	.047
Height SDS	1.2 ± 1.1	0.9 ± 1.2	.35
BMI SDS	1.1 ± 0.9	0.8 ± 1.2	.34
Basal LH (IU/L)	2.2 ± 1.8	1.1 ± 1.1	.018
GnRH stimulated LH (IU/L)	20.4 ± 15.8	14.7 ± 10.1	.26
Basal FSH (IU/L)	4.9 ± 2.7	3.7 ± 1.7	.08
Menarche (years)	10.6 ± 1.7	11.4 ± 1.1	.22
Final height SDS in treated patients	−0.8 ± 1.1	−1.3 ± 0.9	.60
Target height SDS	−0.7 ± 1.0	−0.1 ± 0.9	NA

Data are shown as mean ± standard deviation. The clinical data in this comparison are of the female patients only because male subjects had fewer data available to perform this comparison.

Abbreviations: CA, chronological age; SDS, standard deviation score; FSH, follicle-stimulating hormone; LH, luteinizing hormone; GnRH, gonadotropin-releasing hormone; BMI, body mass index; BAA, bone age advancement; NA, not applicable.

[a]Severe *MKRN3* mutations: p.Pro161Argfs*10; p.Tyr391*; p.Arg213Glyfs*73; p.Asn409Profs*11; p.Gln226Thr fs*6; p.Glu256Gly fs*36; p.Arg189Leufs*17; c.-150_-147delTCAG.

[b]Missense *MKRN3* mutations: p.Arg365Ser; p.Arg328Cys; p.Arg328His; p.Phe417Ile; p.Ala302Val; p.Tyr418His; p.Arg242Trp; p.Phe417Leu; p.Met297Arg; p.Arg68His.

Table 5. Phenotype comparison between girls with CPP with and without MKRN3 mutations

	MKRN3 CPP (n = 45)	Idiopathic CPP (n = 156)	P
Thelarche (years)	6.3 ± 1.2	6.0 ± 1.7	.38
Pubarche (years)	7.1 ± 1.2	6.8 ± 1.9	.24
First evaluation (years)	7.2 ± 1.1	8.4 ± 2.0	.001
Diagnostic delay (years)	0.8 ± 0.8	2.4 ± 2.1	<.001
BAA (years)	2.1 ± 1.6	2.6 ± 1.3	.08
Height SDS	1.2 ± 1.2	1.7 ± 1.1	.04
BMI SDS	0.9 ± 0.9	0.8 ± 0.9	.90
Basal LH (IU/L)	1.7 ± 1.8	1.3 ± 1.4	.22
Basal FSH (IU/L)	4.9 ± 2.3	3.8 ± 2.7	.03
Estradiol (ng/dL)	29.8 ± 20.8	30.1 ± 30.3	.95
LH peak (IU/L)	20.2 ± 14.4	17.3 ± 16.6	.45
FSH peak (IU/L)	16.9 ± 4.6	14.2 ± 10.2	.18

Data are shown as mean ± standard deviation. Diagnostic delay (years) was defined as the difference between the age at first evaluation and the age at fist pubertal signs.

Abbreviations: BAA, bone age advance; CPP, central precocious puberty; SDS, standard deviation score; LH, luteinizing hormone; FSH, follicle-stimulating hormone.

often be difficult to demonstrate or confirm, leading to the misclassification as a sporadic CPP case.

Here, we identified 18 rare inactivating mutations in the *MKRN3*, including 1 nonsense, 6 frameshifts, and 10 missense mutations, along with a promoter region deletion; some of these variants were previously reported (5, 13, 18). Seven are novel variants, including 2 frameshift mutations (p.Arg189Leufs*17 and p.Asn409Profs*11) and 5 missense variants (p.Ala302Val, p.Tyr418His,

p.Arg242Trp, p.Met297Arg, and p.Arg68His). Notably, a recurrent frameshift mutation (p.Pro161Argfs*) was identified in 46% of the patients with CPP. This frameshift affects a cytosine-rich region, confirming this site as a hotspot region.

It is well known that estradiol is the most important hormone in bone maturation, although its serum levels are not good markers of initial pubertal development in girls. While low levels of estradiol in the beginning of puberty

enhance longitudinal growth, the gradually higher estradiol levels towards the end of puberty promote growth plate fusion (19). Curiously, severe mutations were associated with greater bone age advancement and higher basal LH levels, suggesting that these mutations could lead to either prolonged or a greater impact of estradiol levels on bone maturation or more rapid advancement of puberty.

The 10 identified rare missense variants were predicted to be pathogenic by in silico analysis and protein modelling. In addition, 60% of these variants are predicted to lead to loss of stability of the protein. Moreover, 3 variants were located within the C3HC4 ring zinc finger domain of the protein and 4 variants within the third C3H1 zinc finger motif. These domains are related to the E3 ubiquitin ligase activity and RNA -binding, respectively, and appear to be essential for protein function. Recently, Abreu et al. (20) described that MKRN3 acts by repressing the promoter activity of *KISS1* and *TAC3*, which are 2 stimulators of GnRH secretion. Additionally, they found that mutations affecting the RING finger domain led to reduced ubiquitin ligase activity and reduced repression of *KISS1* and *TAC3*. Few studies have explored *MKRN3* regulation so far. Recently, a study demonstrated that leptin, which is a known permissive factor for puberty, does not seem to influence *Mkrn3* expression in mice (21). In addition, the decline of *Mkrn3* expression prior to pubertal onset was also shown to be independent of estradiol (20). At the molecular level, microRNAs appear to have an important role in regulation of *MKRN3* expression. The microRNA miR-30 has been demonstrated to act on 3 binding sites in a highly conserved region of the *Mkrn3* 3′-untranslated region (UTR) as a repressor of *Mkrn3* to control pubertal onset (22). Taken together, our findings might suggest that missense variants could lead to precocious puberty through at least 2 hypothetical mechanisms: (1) destabilizing the protein and generating reduced inhibition of genes that promote puberty, and (2) affecting critical regions (ie, RING fingers) that are relevant to ubiquitination and overall *MKRN3* repressor activity.

Another rarer mechanism has been described in children with CPP who harbored deletions in the 5′-UTR regulatory region of the *MKRN3* gene (13). Subsequently, novel heterozygous mutations (−166, −865, −886 nt upstream to the transcription start site) located in the promoter and 5′-UTR regulatory regions of the *MKRN3* gene were identified in girls with CPP from Cyprus (23). In silico analysis and gene reporter assay for the mutated 5′-UTR predicted a significant change of the mRNA secondary structure and a significant reduction of *MKRN3* promoter activity in transfected GN11 cells, respectively.

Recently, Ramos et al. (24) described anthropometric, metabolic and reproductive outcomes of 11 patients with CPP caused by *MKRN3* mutations who were submitted to GnRH analog treatment. No deleterious effect was evident in young female adults in this specific group of CPP. Interestingly, a high prevalence of overweight and obesity were observed in CPP patients with or without *MKRN3* mutations (47.3% and 50%, respectively), followed by a significant reduction after GnRH analog treatment. Mean final height was similar in CPP groups with or without *MKRN3* mutations, indicating adequate response to GnRH analog treatment in both groups. Here we have further expanded this cohort and the results were consistent with the efficacy of GnRH analog treatment in CPP caused by *MKRN3* mutations.

Our findings demonstrate that the premature sexual development phenotype caused by *MKRN3* loss-of-function mutations is indistinct from idiopathic CPP. Collectively, a shorter time to presentation and higher FSH levels were found in the *MKRN3* patients. Some missense variants lead to destabilization of the protein or affected critical regions within the MKRN3 protein structure. Notably, frameshift mutations and other severe defects have a greater impact on phenotype when compared to pathogenic missense variants. Severe mutations result in greater bone age advancement and higher basal LH levels in patients with CPP.

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4.4 – ARTIGO 3 - THE CONGENITAL AND ACQUIRED MECHANISMS IMPLICATED IN THE ETIOLOGY OF CENTRAL PRECOCIOUS PUBERTY

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The Congenital and Acquired Mechanisms Implicated in the Etiology of Central Precocious Puberty

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Abstract

The etiology of central precocious puberty (CPP) is multiple and heterogeneous, including congenital and acquired causes that can be associated with structural or functional brain alterations. All causes of CPP culminate in the premature pulsatile secretion of hypothalamic GnRH and, consequently, in the premature reactivation of hypothalamic-pituitary-gonadal axis. The activation of excitatory factors or suppression of inhibitory factors during childhood represent the 2 major mechanisms of CPP, revealing a delicate balance of these opposing neuronal pathways. Hypothalamic hamartoma (HH) is the most well-known congenital cause of CPP with central nervous system abnormalities. Several mechanisms by which hamartoma causes CPP have been proposed, including an anatomical connection to the anterior hypothalamus, autonomous neuroendocrine activity in GnRH neurons, trophic factors secreted by HH, and mechanical pressure applied to the hypothalamus. The importance of genetic and/or epigenetic factors in the underlying mechanisms of CPP has grown significantly in the last decade, as demonstrated by the evidence of genetic abnormalities in hypothalamic structural lesions (eg, hamartomas, gliomas), syndromic disorders associated with CPP (Temple, Prader-Willi, Silver-Russell, and Rett syndromes), and isolated CPP from monogenic defects (MKRN3 and DLK1 loss-of-function mutations). Genetic and epigenetic discoveries involving the etiology of CPP have had influence on the diagnosis and familial counseling providing bases for potential prevention of premature sexual development and new treatment targets in the future. Global preventive actions inducing healthy lifestyle habits and less exposure to endocrine-disrupting chemicals during the lifespan are desirable because they are potentially associated with CPP.

Graphical Abstract

Precocious puberty is a prevalent endocrine disorder that affects children worldwide (1, 2). Classically, it is defined as the development of secondary sexual characteristics before the age of 8 years in girls and 9 years in boys, and it has a clear female predominance (3, 4). The premature reactivation of pulsatile hypothalamic GnRH secretion leads to central precocious puberty (CPP), the most common mechanism of precocious sexual development (3).

ESSENTIAL POINTS

1. Central precocious puberty (CPP) results from the premature reactivation of pulsatile hypothalamic GnRH secretion, which can be caused by congenital or acquired disorders with or without central nervous system (CNS) abnormalities.
2. Hypothalamic hamartoma (HH) is the most well-known congenital cause of CPP with CNS abnormalities and its anatomical connection to the anterior hypothalamus, autonomous neuroendocrine activity in GnRH neurons or trophic factors secreted by HH (such as TGF-α), and mechanical pressure applied to the hypothalamus are the main potential mechanisms of HH-related CPP.
3. The identification of monogenic causes of familial CPP, mainly represented by loss-of-function mutations in the maternally imprinted *MKRN3* gene, highlighted novel congenital causes of CPP without CNS abnormalities.
4. Loss-of-function mutations in DLK1, an antiadipogenic factor, in familial CPP were associated with unfavorable metabolic outcome in women, indicating a potential link between the reproductive and metabolic systems.
5. Acquired causes of CPP with CNS abnormalities such as neoplastic lesions, hydrocephalus, neonatal infections, traumatic brain injury, cranial irradiation, and encephalopathies can disrupt the regulatory mechanisms of GnRH release and lead to CPP by poorly established mechanisms.
6. CPP can be associated with multiple anomalies, including growth, metabolic and neurocognitive defects, characterizing syndromic forms of CPP.
7. Endocrine-disrupting chemicals (EDC) have a potential effect in pubertal development through central and peripheral mechanisms and their possible role in human pubertal timing needs to be investigated by well-designed longitudinal studies considering time, duration, dose, and combination of EDC exposure.

The incidence of CPP is 10- to 20-fold higher in females and quite variable in distinct geographical regions, varying from 0.217 to 26.28 per 10 000 girls and 0.02 to 0.9 per 10 000 boys (1, 5–7). These epidemiological data were mainly based on national patient registries, national insurance claims data, and tertiary care centers. A Spanish epidemiological study showed an estimated CPP global prevalence of 19 per 100 000 individuals (girls, 37; boys, 0.46) (8). The annual incidence ranged between 0.02 and 1.07 new cases per 100 000 individuals, with a remarkable increase from 2000 onwards among girls for the period analyzed (1997-2009) (8). A more recent study involving a very large group of children (8596) with premature sexual development suggested that the annual incidence of CPP has substantially increased in Denmark throughout the past 20 years (1998-2017) reaching 9.2 per 10 000 girls and 0.9 per 10 000 boys (1). Similarly, epidemiological longitudinal studies demonstrated a significant increase of CPP in children from Korea (6, 7). The first Korean study (12 351 children) evidenced an increased incidence of CPP from 0.33 per 10 000 girls to 5.04 per 10 000 girls and from 0.03 per 10 000 boys to 0.12 per 10 000 boys between 2004 to 2010 (6). From 2008 to 2014, the incidence of CPP increased from 8.94 per 10 000 girls to 41.53 per 10 000 girls and from 0.16 per 10 000 boys to 1.47 per 10 000 boys (7).

The clinical hallmarks of CPP include progressive breast development in girls and increase of testes volume in boys and reflect GnRH and gonadotropin-stimulated sex steroid actions (gonadarche) (3, 9). Accelerated growth velocity (>6 cm/y) and advanced bone age (higher than 1 year or 2 SD score of chronological age) represent common features of progressive CPP. Hormonal findings confirming diagnosis of CPP include pubertal basal or GnRH-stimulated LH levels (3). The presence of family history or CPP associated with multiple anomalies can suggest a genetic etiology (3). The prevalence of familial CPP was 27.5% in a large cohort of 153 children with idiopathic CPP (10). In the recent past, magnetic resonance imaging (MRI) of the central nervous system (CNS) was the only approach in the investigation of the etiology of CPP, searching mainly for congenital or acquired anatomical alterations. More recently, genetic studies were added to the algorithm of etiology investigation of familial or sporadic CPP in children without CNS anatomical lesions (3).

Comprehension of the mechanisms underlying CPP expanded significantly in the past 2 decades, by using refined clinical approaches, updated genetic studies, and neuroimaging (11). Distinct genetic approaches ranging from genome-wide association studies, linkage analysis, comparative genomic hybridization, and whole-exome and genome sequencing have allowed for the identification of new players implicated in not only variations within normal puberty timing, but also within the etiology of progressive CPP (12, 13). Monogenic causes of familial

CPP, including syndromic and nonsyndromic forms, have helped reveal novel signaling pathways implicated with normal and abnormal pubertal timing in humans (14–16). The genetic discoveries involving the etiology of CPP have had an important influence on the diagnosis (more precise and earlier) and familial counseling and can provide the bases for potential new treatment targets in the future. Additionally, the involvement of metabolic alterations (overweight/obesity, metabolic syndrome) and endocrine-disrupting chemicals (EDC) exposure were added to our current knowledge on the etiology of CPP (17).

Several factors could influence the timing and tempo of puberty, including genetics, lifestyle, nutrition, and environmental exposures (11, 18, 19); however, the mechanisms underlying the increasing trend in the incidence of CPP are uncertain. The growing influence of nutritional status (overweight or obesity) has been highlighted as a major influence on premature pubertal development, especially in girls (20). Other potential mechanisms include prenatal and postnatal exposure to endocrine disruptors, international adoption, use of electronic devices, and psychosocial influences (21–24). An increased incidence of precocious and accelerated puberty was demonstrated in a small cohort of Italian girls during and after lockdown for the coronavirus disease 2019 (23, 25). This was potentially related to weight gain, more frequent use of electronic devices, and stress (25). Notably, earlier age at puberty has been associated with a higher risk of metabolic, oncologic (estrogen-dependent cancer in women in adulthood), and cardiovascular disorders in adulthood (26). It has been hypothesized that the increasing prevalence and progression of CPP may also represent an adaptive mechanism to escape from ectopic adiposity in girls, especially in those who had higher abdominal fat distribution (27).

Recognition of the causes of CPP has contributed to our understanding of the potential mechanisms responsible for normal and abnormal pubertal timing, allowing the development of novel strategies, including prophylactic intervention, diet alterations, and behavior targeted therapies, increased surveillance, and delivery of personalized care. Here, we present a comprehensive review on the multiple known CPP etiologies, exploring the status of the studies aimed at untangling the underlying mechanisms implicated in the premature hypothalamic GnRH secretion.

Neurobiological Basis of Puberty

The hypothalamus is the main control center for different physiological functions, including growth, metabolism, and puberty/reproduction. It is responsible for the pulsatile GnRH secretion into the hypophyseal portal blood system that in turn controls the release of gonadotropins, LH and FSH, from the anterior pituitary to drive gonadal maturation and function. These gonadotropins participate in the maturation and functioning of the gonads (ie, testis and ovary) that are responsible for the synthesis of sex hormones (28), thus promoting the development of secondary sex characteristics (9) and the production of gametes. What signals the activation of this axis to incite pubertal onset has been a long-standing enigma in neuroendocrinology. Clinical observations of pubertal phenotypes lead to the identification of specific allelic variants in genes coding factors such as kisspeptins, neurokinin-B (NKB), and leptin that were later experimentally shown to be key players in mammalian puberty (29).

The *KISS1* gene encodes the kisspeptins that bind to and activate their specific receptor KISS1R (previously known as GPR54) (30). The identification of loss-of-function mutations in KISS1R, a G-protein coupled receptor, in families with congenital hypogonadotropic hypogonadism was a critical discovery in the field of neuroendocrine regulation of reproduction (30, 31). Analyses of the reproductive and hormonal phenotypes of *Kiss1r* null mice and humans revealed that hypothalamic GnRH synthesis and neuronal migration, and pituitary responsiveness to GnRH, were preserved, suggesting that this system is an upstream regulator of GnRH release (32). Kisspeptins were originally identified as metastasis suppressor peptides (33). Physiological and pharmacological studies have shown that the kisspeptins ligand/receptor system is an essential part of the excitatory network that regulates GnRH secretion. Later, very rare inactivating *KISS1* mutations were also identified in patients with congenital hypogonadotropic hypogonadism, underscoring the importance of kisspeptins for puberty and reproduction in humans (34).

Multiple human, animal, and in vitro studies have shown that GnRH secretion requires the stimulatory action of kisspeptins (35–37). KISS1 and KISS1R are broadly expressed in human and animal tissues, with high expression in the placenta and several brain areas, including hypothalamus. In the hypothalamus, kisspeptins is expressed in the arcuate nucleus (ARC) of male and female rodents, and in the anteroventral periventricular nucleus/periventricular nucleus continuum (AVPV) of female rodents. Kisspeptins neurons in the ARC coexpress the neuropeptides, NKB (encoded by *TAC3* gene) and dynorphin A (called KNDy neurons) (38, 39). In these KNDy neurons, the coordinated action of NKB (stimulatory) and dynorphin (inhibitory) controls release of kisspeptins to effect pulsatile GnRH and LH secretion (40). By contrast, kisspeptins neurons in the AVPV/periventricular nucleus continuum are involved in the positive feedback of sex steroids, leading to the preovulatory LH surge in females mice (41–43). Administration of kisspeptins results in increases in plasma LH concentrations in healthy men (36, 44), and in women kisspeptins also induce LH release, although the response varies across the menstrual cycle (45). Kisspeptins stimulate gonadotropin release less potently but in a more physiologically effective way than current treatments with GnRH analogues (35).

Hypothalamic kisspeptins neurons serve as the nodal regulatory center of reproductive function (46). As stated previously, kisspeptin expression, synthesis, and release are tightly regulated by metabolic cues at multiple levels (47). Agouti-related protein and pro-opiomelanocortin neurons are critical components of neuroendocrine circuits regulating activity of kisspeptins neurons (48), as well as additional hypothalamic centers that have also been identified as regulators of these neurons (47, 49). Although peripheral signals transmit fundamental metabolic information to achieve successful reproduction, kisspeptins neurons do not seem to be first-order responders for the main metabolic cues, such as leptin, insulin, and ghrelin (47).

The coordination of energy intake and expenditure is a complex process that is orchestrated by specific neuronal populations in the hypothalamus and influenced by both peripheral and central signals that ultimately regulate body weight. Glial cells have been recognized as important protagonists in

this neuroendocrine process (50). Metabolic hormones, such as leptin and ghrelin, and specific nutrients relay information regarding nutritional status to hypothalamic neuronal circuits to determine appetite and energy expenditure. Some of these metabolic and nutritional factors, as well as neuropeptides controlling energy homeostasis, also participate in the control of puberty (29). The interaction of these 2 physiological axes is clearly observed at the 2 extremes of body energy status: chronic energy deficiency, such as malnutrition or anorexia, which can be accompanied by delayed puberty or lack of pubertal progression, whereas the opposite condition of energy excess, as seen in obesity, has been associated with early pubertal onset (17, 51). This phenomenon of nutritional effects on pubertal development has been extensively documented in girls (52), but it may also occur in boys (53).

There is a clear relationship between nutrition, the timing of pubertal onset and its progression, and linear growth (54). Nutritional status plays an important role in regulating growth, and excess body weight/adiposity early in life (11, 54). Deardorff et al (55) demonstrated the relationship between childhood overweight and obesity and pubertal onset among Mexican-American boys and girls by using data from the Center for the Health Assessment of Mothers and Children of Salinas (55). This study investigated the association between body mass index (BMI) at age 5 years and multiple markers of pubertal onset in 336 Mexican-American children. No association between these factors was observed in boys, but it found significantly earlier thelarche in overweight and obese girls, menarche in overweight girls, and pubarche in obese girls, as compared to normal-weight girls (55). Martos-Moreno et al (56), analyzing data from the Madrid Cohort of Pediatric Obesity, also demonstrated a strong relationship between sex, ethnicity, growth, nutrition, and puberty. Ethnicity is one of the main determinants of adipose tissue distribution with increased trunk body fat accumulation in Latino children with obesity, which is directly related to the development of metabolic derangement (57). The age of menarche, which occurs approximately 2 years after the onset of thelarche, correlates positively with bone age and negatively with the amount of growth that remains to occur (58). The connection between metabolic status and puberty has not only been found in clinical studies, but it has also been clearly demonstrated in preclinical studies employing animal models of early under- or overnutrition where pubertal timing is delayed or advanced, respectively (17). These studies provided a valuable tool to discern the mechanisms involved in pubertal disorders caused by metabolic distress (59).

The cellular energy sensors mammalian target of rapamycin (mTOR), AMP-activated protein kinase, and the sirtuin, are also involved in the metabolic regulation of puberty (17). In the hypothalamus, mTOR and AMP-activated protein kinase signaling operate in a reciprocal manner to promote or repress puberty, respectively, via activation or inhibition of Kiss1 neurons in the arcuate nucleus depending on energy status (60, 61).

In addition, brain ceramides have been proposed as potential mediators in the control of energy balance in adult rodents. They mediate the orexigenic actions of ghrelin and oppose the anorectic effects of leptin, as well as control brown fat activity (62). Heras et al (62) demonstrated that female rats with early-onset overweight not only presented advanced

puberty, but that this was associated with an enhancement of hypothalamic ceramide content and that central pharmacological activation of ceramide signaling mimicked the advancement of puberty caused by obesity (62). These data clearly support the concept of a tight relationship between energy homeostasis and puberty, with a putative role of cellular energy sensors and metabolic mediators in the control of puberty (Fig. 1).

Menarche is an important and specific marker of pubertal timing in females. Perry et al (12) described that menarche signals are enriched in imprinted regions, with 3 *loci*, *MKRN3/MAGEL2,* Delta-like Noncanonical Notch Ligand 1 (*DLK1*)/*WDR25*, and *KCNK9*, showing parent-of-origin specific associations concordant with known parental expression patterns. Indeed, loss-of-function mutations in *MKRN3* and *DLK1* have been associated with CPP in several families, as described in the following section (14, 63). Subsequent studies in a normal population using expanded genomic analysis identified a rare variant in the *MKRN3* 5′ untranslated region (UTR) (rs530324840) and another variant in the *MKRN3 locus* (rs530324840), which had significant associations with the age of menarche (12).

In mice, *MKRN3* expression is higher in the hypothalamus compared with cortical brain, liver, and testis before sexual maturation, and expression in the hypothalamus and brain declines with pubertal development, as in rats and female nonhuman primates (64). The decline of *MKRN3* expression before sexual maturation in mice is independent of changes in sex steroids and leptin, indicating that *MKRN3* regulation is upstream in the HPG axis (64, 65). *MKRN3* is expressed in KNDy neurons and inhibits transcription of 2 important GnRH stimulators, *KISS1* and *TAC3*, in vitro (64). Hypothalamic kisspeptins mRNA levels are maximum around the time of puberty in both male and female rats (66). Similarly, *Tac3* expression also increases during pubertal development in the ARC in mouse, with the time course of the increase strikingly parallel to the decrease in *MKRN3* expression (14, 67). Notably, *MKRN3* did not alter promoter activity of prodynorphin (*PDYN*), the gene encoding the inhibitory peptide dynorphin, which is coexpressed in KNDy neurons. Taken together, these data point to an important role of *MKRN3* in KNDy neurons, and in association with *MKRN3* loss-of-function variants in precocious pubertal development in humans, indicates that *MKRN3* is a component of the inhibitory network suppressing the HPG axis during childhood (14, 64).

MKRN3 is an E3 ubiquitin ligase and undergoes autoubiquitination (64). *MKRN3* regulates *GnRH1* transcription through ubiquitination of methyl-CpG-DNA binding protein 3, disrupting its binding to the *GnRH1* promoter (68). In addition, ubiquitination of poly A-binding proteins by MRKN3 leads to shortening of the poly A tail length of *GnRH1* mRNA, compromising the formation of the translation initiation complex (69). These data indicate that MKRN3 can control both transcriptional and posttranscriptional switches of pubertal initiation.

Little is known about specific genes that regulate male puberty. In boys, some BMI-increasing alleles have been shown to associate with earlier, and others with delayed, sexual development; these genetic results mimic the controversy in epidemiological studies, some of which show opposing correlations between prepubertal BMI and male puberty (70).
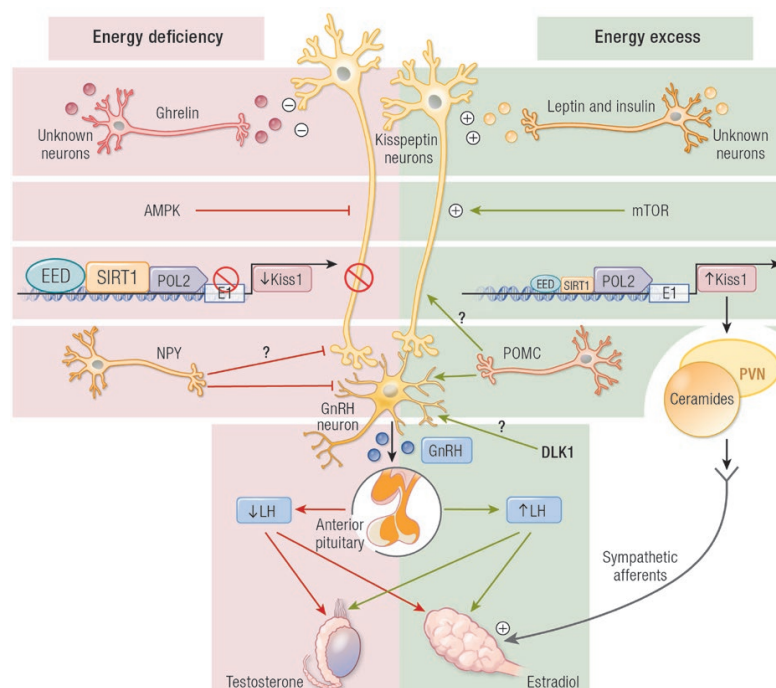
Figure 1. Schematic representation of the putative role of cellular energy sensors and metabolic mediators in the control of puberty. In conditions of energy insufficiency, hypothalamic activation of AMP-activated protein kinase (AMPK), together with persistence of the repressive action of SIRT1 at the Kiss1 promoter, leads to reduced Kiss1 expression and delayed puberty. By contrast, in conditions of energy sufficiency and timely removal of SIRT1 from the Kiss1 promoter, together with the presumable activation of mammalian target of rapamycin (mTOR), allows increased Kiss1 expression and the normal occurrence of puberty. At extreme conditions of energy excess (eg, early-onset obesity), precocious removal of SIRT1 from the Kiss1 promoter causes a change in the chromatin landscape that accelerates the rise in Kiss1 expression, leading to early puberty. In addition, obesity presumably also causes the activation of an alternative pathway, involving kisspeptins innervation of the paraventricular nucleus (PVN) and ceramide (CER) synthesis at this site, which apparently contribute to the precocious activation of the ovary in obesity via direct sympathetic inputs. ARC, arcuate nucleus; Dyn, dynorphin; GnRH, gonadotrophin-releasing hormone; Kp, kisspeptins; mTOR, mammalian target of rapamycin; NKB, neurokinin B; SIRT1, Sirtuin; SNS, sympathetic nervous system.

Although the mechanisms regulating pubertal onset may largely be shared in males and females, the relationship between body mass and pubertal timing in boys may be more complex than previously thought and requires further genetic studies.

Ethnic Influences on Human Puberty

The timing of normal pubertal onset is proposed to be influenced by ethnicity, especially in girls. The first study designed to analyze this possibility was performed by Harlan et al (71) and published in 1980; indeed, despite the grand ethnic diversity found in this country, as well as others, the possible ethnic influence on puberty has only been seriously addressed in recent decades. The data of this seminal study (71) suggested that Afro-American girls enter puberty earlier and reach menarche at a younger age than Caucasian and Hispanic girls. It was not until 2002 that it was shown that non-Hispanic Black girls and boys were shown to mature

earlier than Mexican American or non-Hispanic White children (72). The authors of this later study indicated that the national reference data in the United States for the timing of sexual maturation should take this information into consideration to appropriately interpret the normal age of sexual maturation in US children. Despite few confirmatory studies (73, 74), it is generally accepted that African American girls enter pubertal development before Caucasian and Hispanic girls (75).

Unfortunately, there are limited data available regarding pubertal timing that takes ethnical diversity throughout the world into consideration. However, most pediatric endocrinologists would agree that the reported ethnical differences regarding pubertal timing are derived from a mixture of genetic, social, and environmental factors that are heterogenous within and between populations (75, 76), as well as the interaction between these different elements.

A recent systematic review and meta-analysis found that age at pubertal onset, with thelarche assessed by physical or

clinical examination of the breast, decreased by a mean of almost 3 months per decade from 1977 to 2013 (77). Thus, in function of the criteria employed, the diagnosis of CPP will clearly be affected as well as the subsequent treatment (78).

Multiple Etiologies of CPP

CPP can result from congenital (genetic and nongenetic causes) or acquired CNS lesions (3). Patients with CPP can be classified as familial or sporadic cases, and as syndromic or nonsyndromic forms (3, 11). If the underlying causes are not identified, CPP is classified as idiopathic, the most prevalent form in the female sex (3). According to epidemiological studies, the prevalence of CPP is up to 23 girls for every boy (79). Progress in the genetic investigation of patients with familial or sporadic CPP, presently classified as idiopathic, will reduce the prevalence of the idiopathic form in both sexes in the future.

A refined etiological classification of several CPP causes is suggested in this review, aiming to explore the underlying putative mechanisms of the premature reactivation of the HPG axis (Table 1). We have divided the current causes into 2 major groups: congenital or acquired causes. Both groups are then divided into 2 other branches, characterized by the presence (or absence) of structural brain lesions. Another fundamental component of this classification involves the recognition of signs of syndromic features associated with CPP, characterized by a more complex developmental phenotype in the congenital causes (Table 2). The identification of familial CPP or gene alterations can be relevant in the diagnosis of a congenital disease, such as monogenic or syndromic CPP with distinct inheritable patterns that would promote an active surveillance of new cases in the same family.

Congenital Etiology of CPP

Congenital Causes With CNS Lesions

The most common congenital cause of CPP associated with CNS lesions in both sexes is hypothalamic hamartoma (HH), which is implicated in early HPG axis reactivation by several putative mechanisms. A systematic review and meta-analysis including 15 studies and 1853 girls identified 9% of CPP patients with CNS alterations in MRI, with HH representing 40% of these lesions (80).

The overall prevalence of intracranial pathology (congenital and acquired) associated with CPP range from 0% to 24.3% in girls, and up to 74% in boys, with congenital causes representing the greatest prevalence in both sexes (80–82). If considering only girls aged <6 years, the prevalence of intracranial pathology increases up to 29% (80). The prevalence of CNS lesions in boys with CPP was revised in distinct ethnical groups. A Turkish study including 100 boys with CPP identified only 26% with CNS lesions, indicating that the number of idiopathic male CPP cases is increasing over time (83). In addition, Chinese and Taiwan studies demonstrated a low prevalence of lesions in CPP male cohorts (16.3% and 7%, respectively) (84, 85). More recently, a high prevalence (40%) of monogenic causes (loss-of-function *MKRN3* mutations) were found in 20 boys with familial CPP without CNS lesions, suggesting the importance of genetic analysis in males (86).

There is a lack of consensus whether some congenital abnormalities, such as Rathke cleft cysts, Chiari malformation, and pineal and arachnoid cysts, are definitive causes of CPP. Some investigators considered them as incidental findings (87), whereas others as potential causes of CPP based on epidemiological evidence (88). CNS lesions with questionable

Table 1. Etiology of CPP: congenital and acquired causes with or without CNS lesions

1–Congenital	2–Acquired
1A–with CNS lesions	**2A–with CNS lesions**
• Hypothalamic hamartoma (HH)	• **Tumoral causes**
• Neurofibromatosis type 1 (NF1)	Astrocytoma, ependymoma, pinealoma, hypothalamic or optic pathways glioma, craniopharyngioma, dysgerminoma (non-hCG secreting), meningioma
• Arachnoid cysts	• **Post-insults**
• Meningomyelocele/Chiari malformation type 2	Neonatal infections, granulomatosis disease, cerebrovascular accidents, hydrocephalus, traumatic brain injury, cranial irradiation, encephalopathies
Other conditions:	• **Cerebral palsy**
• Hydrocephalus and/or meningomyelocele	
• Chiari type 1 malformation	
• Septo-optic dysplasia	
• Tuberous sclerosis complex	
• Duplication of the pituitary gland	
• Other rare syndromic forms of CPP	
1B–without CNS lesions	**2B–without CNS lesions**
• Gain-of-function mutations in the genes encoding kisspeptin (*KISS1/KISS1R*)	• Endocrine-disrupting chemicals
• Loss-of-function mutations in Makorin ring finger 3 (*MKRN3*)	• International adoption
• Loss-of-function mutations in delta-like-homolog type 1 (*DLK1*)	• Early exposure to sex steroids
• Syndromic CPP	

Table 2. Genetic and epigenetic syndromes associated with central precocious puberty

Disorder (OMIM)	Critical region	Main molecular diagnosis	Prevalence of CPP	Other main clinical features	Putative mechanism(s) or gene involved in CPP
Syndromic CPP without CNS lesions					
Temple syndrome (616222)	14q32.2	1. UPD(14)mat 2. *DLK1/MEG3*:IG-DMR hypomethylation 3. 14q32.2 paternal deletion	80%–90%	Prenatal and postnatal growth failure, hypotonia, small hands and/or feet, obesity, motor delay	*DLK1*
Prader-Willi syndrome (176270)	15q11-q13	1. 15q11-q13 paternal deletion 2. UPD(15)mat	4%	Hypotonia, obesity, growth failure, cognitive disabilities, hypogonadism	*MKRN3*
Silver-Russell syndrome (180860)	11p15.5	*IGF2/H19*:IG-DMR hypomethylation	Overall: 5-15% UPD(7)mat: likely higher prevalence	Prenatal and postnatal growth retardation, relative macrocephaly, prominent forehead, body asymmetry, feeding difficulties	11p15.5 defects: not established
	Not established (chromosome 7)	UPD(7)mat			UPD(7)mat: possible imprinted or recessive factors to be elucidated
Williams-Beuren syndrome (194050)	7q11.23	Hemizygous 7q11.23 deletion	3%-18%	Distinct face, cardiovascular disease, short stature, intellectual disability, hypersociability	Contiguous gene syndrome CPP mechanism remains unclear
Xp22.33 deletion (*SHOX* region)	Xp22.33	Xp22.33 deletion with pseudo-autosomal dominant inheritance, involving *SHOX*	Rare cases	*SHOX* phenotypes: body disproportion, short stature, Madelung deformity	CPP mechanism remains unclear
Xp11.23-p.11.22 duplication syndrome (300881)	Xp11.23-p11.22	Xp11.23-p11.22 duplication with X-linked dominant inheritance	Females: 70% Males: 11%	Intellectual disability, speech delay, electroencephalogram abnormalities, excessive weight, skeletal anomalies	Contiguous gene syndrome. CPP mechanism remains unclear
MECP2 defects (300005)	Xq28	Defects with X-linked dominant inheritance: 1. *MECP2* loss-of-function mutations 2. Xq28 duplication involving *MECP2*	Rare cases of atypical Rett syndrome	Neurodevelopmental phenotypes, intellectual disability, autism	*MECP2*
X-linked intellectual developmental disorder Snijders Blok type (300958)	Xp11.4	X-linked dominant de novo mutations in *DDX3X* affecting females	Females: 13%	Intellectual disability, developmental delay, hypotonia, behavior problems, movement disorders, skin abnormalities	*DDX3X*
Kabuki syndrome (147920)	12q13.12	Loss-of-function mutations in *KMT2D*	Premature thelarche: 40% CPP: uncommon	Neurodevelopmental phenotypes, typical distinct face, short stature, multiple anomalies	Possible downregulation of estrogenic receptor activation
Mucopolysaccharidosis type IIIA or Sanfilippo disease (252900)	17q25.3	Homozygous or compound heterozygous mutations in *SGSH*	Very rare (5 boys)	Severe neurologic deterioration, visceromegaly, skeletal abnormalities	Possible accumulation of glycosaminoglycans triggering GnRH

Rare cases of distinct copy number variants: 1p36 deletion ([14](#)), 9p distal deletion ([15](#)), and 9q34.3 duplication (including *NOTCH1*) ([16](#)).

Table 2. Continued

Disorder (OMIM)	Critical region	Main molecular diagnosis	Prevalence of CPP	Other main clinical features	Putative mechanism(s) or gene involved in CPP
Syndromic CPP with CNS lesions					
Pallister-Hall syndrome (146510)	7p.14.1	Heterozygous pathogenic variant in GLI3	Unknown	HH, mesoaxial polydactyly, panhypopituitarism, imperforate anus and other visceral anomalies	Hypothalamic hamartoma
Neurofibromatosis type 1 (162200)	17q11.2	Heterozygous pathogenic variant in NF1	72%	Multiple café au lait spots, axillary and inguinal freckling, multiple cutaneous neurofibromas, iris Lisch nodules, and choroidal freckling, learning disabilities, optic nerve and other central nervous system gliomas, malignant peripheral nerve sheath tumors, scoliosis, tibial dysplasia, and vasculopathy	Optic pathway glioma or hamartoma
Tuberous sclerosis complex (191100 and 613254)	9q34, 16p13	Loss-of-function mutation in TSC1, Loss-of-function mutation in TSC2	Rare	Seizures, intellectual disability, and facial angiofibromas	Giant cell astrocytoma or hypothalamic hamartoma

relationship with CPP have been counted in the prevalence data of some studies, impairing the estimation of the real prevalence of pathological CNS lesions associated with CPP (80, 89, 90).

Prevalence of incidental findings in MRI studies of patients with CPP are quite variable (ranging from 2.7% to 9.6%) and include distinct conditions, such as pineal cysts, pituitary microadenomas, pituitary enlargement, pituitary asymmetry, absent septum pellucidum, variation of perivascular space (normal), nonspecific white matter lesion, and hyperintense thalamic lesions (87). Of note, pineal cysts, classified as an incidental finding in CPP patients in several studies (80, 87, 89), are usually asymptomatic with a prevalence of 1.9% in the childhood population (88).

Hypothalamic Hamartoma

HH are rare nonneoplastic benign lesions constituted of ectopic hypothalamic tissue typically located at the base of the cranium, under the third ventricle, in proximity to the tuber cinereum and the mammillary bodies (91–93). They are usually small lesions, measuring between 0.5 and 2 cm in diameter, which as a rule remain unchanged over time. HH can occur as an isolated lesion or as part of a syndrome. Clinically, HH can be asymptomatic and if symptomatic both endocrine and neurological manifestations can be present with a heterogeneous spectrum. In this hypothalamic malformation, CPP manifests at a very early chronological age (mean age of 2.5 years in girls and 3.7 years in boys) (91).

Neurological symptoms associated with HH characteristically include gelastic (brief spells of laughter) or dacrystic (crying) seizures, usually presenting during infancy, with later occurrence of different seizure types, such as focal and generalized seizures associated with an epileptic encephalopathy. Developmental delay, loss of acquired developmental milestones, and behavioral disturbances frequently accompany the more severe scenario (94).

The anatomy of the HH as revealed by MRI is predictive of the clinical syndrome and it is appropriate to consider 2 recognized clinicopathological subtypes: HH that are functionally connected to the pituitary stalk and tuber cinereum can cause CPP, whereas those functionally connected to the region of the mammillary bodies and limbic circuit result in epilepsy (91).

The potential mechanisms for the formation of HH as well as the CPP caused by them are not completely known (95). Considered as a model for studying the onset of puberty, HH can accelerate sexual development by producing bioactive substances that mimic a cascade of events that trigger the onset of puberty (96). It has also been suggested that HH that manifest CPP contains intracellular signaling networks and transcription factors essential for pulsatile secretion of GnRH. Both the presence of GnRH-secreting neurons within the HH, as regulatory neurons connected to GnRH neurons in the HH or to hypothalamic neuronal networks, including astrocytic and ependymal cells in the HH tissue, may be necessary for the physiopathological condition of HH-related CPP (95, 96). It is postulated that CNS developmental abnormalities that result in the formation of HH originate from sporadic defects that affect genes of morphogenic pathways involved in the embryonic development of the ventral hypothalamus and third ventricle (97).

Through immunohistochemical studies, the detection of the presence of GnRH-secreting neurons in some HH led to

the concept that HH advances puberty by functioning as an ectopic GnRH-releasing pulse generator (95, 96). The study of 2 HH associated with CPP in females revealed that neither of them contained GnRH neurons, but astroglial cells expressing TGF-α and its erbB-1 receptor (96). TGF-α is a member of the epidermal growth factor family that mediates the facilitating effect that glial cells exert on GnRH neurons. Therefore, some HH can induce CPP, not through the secretion of GnRH, but through the synthesis of trophic factors, such as TGF-α, capable of activating a normal neuronal network of GnRH (96, 97). Interestingly, most of the resected HH tissue from subjects with or without a history of CPP did not express kisspeptins or mRNA of *KISS1R* (98).

The GnRH-secreting neurons in the mediobasal hypothalamus receive abundant GABAergic innervation (91, 99) and those with a wide projection in HH tissue are innervated by GABAergic interneurons. In contrast to the classical inhibitory GABAergic effect on mature GnRH neurons, in immature cells GABA promotes GnRH release, as demonstrated by electrophysiological and pharmacological studies in animal models (100). This suggests the hypothesis that the pulsatile release of GnRH could be related to the GABA excitatory activity in HH (100–102). A schematic representation of the potential mechanisms of CPP caused by HH is presented in Fig. 2.

Considering that HH is the consequence of a defect in the normal embryonic hypothalamic developmental process, that germline mutations in *GLI3* constitute the genetic basis of Pallister-Hall syndrome, which includes HH, and that Gli3

is a regulatory protein of the Sonic Hedgehog (SHH) morphogenetic pathway, it was hypothesized that defects in genes encoding other proteins of the SHH pathway, such as *PRKACA, SMO, CREBBP,* and *GLI2,* could be the genetic basis of nonsyndromic HH (103). The SHH signaling pathway plays an essential role during vertebrate embryonic development and in tumorigenesis (104).

Using a candidate gene study approach, Saitsu et al (105) studied peripheral leukocyte and HH tissue DNA of 18 patients with HH (5 patients with additional syndromic features). Inactivating somatic mutations in 2 genes, *GLI3* and *OFD1,* were identified in Pallister-Hall syndrome and type I orofacial-digital syndrome, respectively (105). In this study, 2 other candidate genes, *UBR5* and *ZNF263,* were proposed (105).

The multicenter study by Hildebrand et al (103) included 38 cases with HH and gelastic epilepsy, of whom 24 had intellectual deficit and 15 had CPP without other syndromic signs. Fourteen somatic defects (4 nonsense mutations, 2 frameshift mutations, 1 insertion, and 7 copy number variants [CNVs] or loss of heterozygosity) in genes involved in the regulation of the SHH pathway were identified in 14 of 38 (37%) patients (103). Four patients with somatic point mutations in *GLI3*, 1 patient with a CNV in *GLI3* and 3 patients with somatic mutations in *PRKACA* represented the most relevant findings of this study. These data implicate defects in the SHH pathway in the pathogenesis of HH associated with epileptic syndrome. Furthermore, *PRKACA*
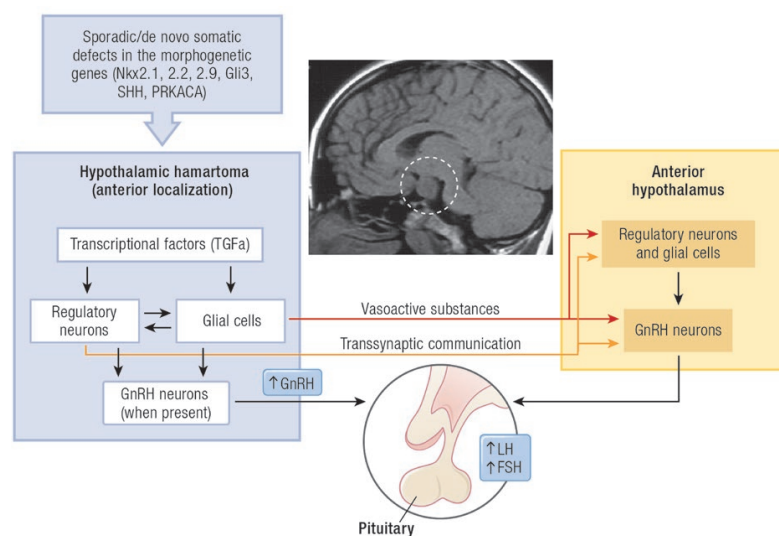
Figure 2. Schematic pathophysiology of hypothalamic hamartoma-related CPP. Sagittal MRI view revealing a large parahypothalamic hamartoma (dotted white circle) attached to anterior hypothalamus as cause of CPP. The HH formation may be determined by sporadic/de novo somatic mutations in genes required for hypothalamic morphogenesis. Several genes, most of them encoding proteins of the Sonic hedgehog (SHH) pathway, have been implicated in the development of syndromic and nonsyndromic hypothalamic hamartoma (HH). The underlying mechanisms leading the ability of HHs to activate GnRH secretion and induce CPP are multiple, including autonomous secretion of GnRH by HH, *trans* synaptic activation, via myelinated fibers, connecting the HH to the hypothalamus, and the secretion of glial products and bioactive substances (TGF-α and downstream factors) capable of stimulating a neuronal network involved in the GnRH secretion. In addition, the effect of the mechanical pressure that HH could apply to the hypothalamus could represent another potential mechanism. There is evidence that the anatomical position (anterior localization) of HH has a pivotal role for the occurrence of CPP.

is currently associated with the pathogenesis of HH (103). However, the patients who manifested CPP were not analyzed separately, and consequently the potential mechanisms involved in this endocrine manifestation were not discussed. Recently, novel pathogenic germline or somatic variants in *DYNC2H1*, *KIAA0556*, and *PTPN11* genes were identified in patients with HH-related epilepsy, with or without CPP, suggesting that disruption of the SHH signaling pathway associated with cilia or the RAS/MAPK pathway may lead to the development of HH (106).

Neurofibromatosis type 1 (NF1)

NF1 is an autosomal dominant multisystemic neurocutaneous disorder characterized by increased risk of benign and malignant tumor formation affecting primarily skin, bone, and the CNS. NF1 is caused by loss-of-function mutations in the *NF1* tumor suppressor gene located on chromosome 17q11.2 (107, 108), which encodes the neurofibromin protein. Comprising more than 2800 amino acids (~220 KDa), neurofibromin contains a small domain (280–300 amino acids) that is structurally and functionally similar to a family of proteins that function as negative RAS regulators (109). Because increased RAS activation is associated with numerous human cancers (110), individuals with NF1 are predisposed to a range of tumors affecting the central and peripheral nervous systems, including optic pathways glioma (OPG), which are a source of significant morbidity in this population (111).

CPP has been reported primarily in NF1 children with OPG with an estimated prevalence of 3% (112). This observation is consistent with the theory that lesions located close to the hypothalamus interfere with tonic CNS inhibition of the HPG axis, resulting in the premature onset of puberty. In several studies, CPP developed exclusively in those patients with NF1 who had OPG involving the optic chiasm (113–117). However, CPP in the NF1 population in the absence of OPG has been described by some authors (118). It was hypothesized that mild cerebral abnormalities, undetectable with MRI, such as slow-growing hamartomas, may lead to CPP in NF1 children without OPG (112). Considering that neurofibromin is part of a signal transduction chain extending from extracellular signals to transcriptional regulation in the nucleus, an abnormal signal transmission pathway could represent a potential underlying mechanism of tumorigenesis (119). Almost all NF1-OPGs) are benign pilocytic astrocytomas (World Health Organization grade I astrocytomas) that can arise anywhere along the optic pathway, including the optic nerves, optic chiasm, optic tracts, and optic radiations (111, 120). However, in individuals with NF1, the majority (75%–85%) of OPGs are located within the optic nerve and chiasm (prechiasmal or anterior optic pathway), with a smaller proportion of tumors located in the optic tracts and radiations (postchiasmal or posterior optic pathway). NF1-OPGs occur most frequently in young children (median age at diagnosis, 4.5 years) (115, 121), with rare cases described in older adolescents (122, 123). Notably, some patients may have both conditions during follow-up, evolving from precocious to delayed/absent puberty (124).

The latter evidence suggests that continued monitoring of individuals with NF1 into adulthood for the development of OPGs and for progression of known OPGs is warranted.

Other CNS Conditions

Other congenital lesions associated with CPP are suprasellar arachnoid cysts, hydrocephalus, tuberous sclerosis, septo-optic dysplasia, Chiari II malformations, and myelomeningocele. Intracranial arachnoid cysts are benign, nongenetic developmental cavities that contain clear secretions similar in nature to cerebrospinal fluid and are completely situated within the arachnoid membrane. Arachnoid cysts are relatively rare, usually congenital, but may also arise after an infection, trauma, or hemorrhage (88). Suprasellar arachnoid cysts account for approximately 10% of all arachnoid cysts and occur almost exclusively in children, somewhat more frequently in boys than in girls. Neurological and visual field abnormalities have been reported in 25% to 85% of patients with suprasellar arachnoid cysts, as well as diverse endocrinological disorders, most notable CPP in 10% to 40% of these children (88, 125, 126). Arachnoid cysts may cause a wide spectrum of endocrinological disorders such as deficiencies of GH, TSH-releasing hormone, and ACTH, but stimulates the HPG axis leading to CPP. These hormone disorders are all due to the proximity of the cyst to the hypothalamic-pituitary area, but their direct mechanisms are unknown (88, 127).

Meningomyelocele, the commonest type of spina bifida, occurs because of abnormal development of the neural tube and manifests as failure of the complete fusion of posterior arches of the spinal column, leading to dysplastic growth of the spinal cord and meninges. Children with meningomyelocele have an increased incidence of CPP (128). Epidemiological studies showed that the prevalence of CPP in girls with meningomyelocele is around 50% and ranges from 10% to 30% in boys (129–131). Although the exact causative mechanism of CPP in children with meningomyelocele is unknown, several studies demonstrated an association with hydrocephalus, which may alter HPG axis function. Additionally, evidence shows that increased perinatal intracranial pressure and brainstem malformations, such as Chiari II malformations, which involves both the cerebellum and brain stem tissue pushing into the foramen magnum, are influential prognostic factors for the development of CPP (128).

Type I Chiari malformation is a disorder characterized by a displacement of the cerebellar tonsils through the foramen magnum into the upper cervical spinal canal without myelomeningocele. Patients with type II Chiari malformation with meningomyelocele can frequently present with CPP, whereas very rare reports show an association between type I Chiari malformation and early puberty (132). The development of CPP in patients with type I Chiari malformation was associated with an increase in peri- and postnatal intracranial pressure because of impaired cerebrospinal fluid circulation or compression and distortion of the hypothalamus (133).

Septo-optic dysplasia is a heterogeneous congenital condition defined by the presence of 2 or more features of the triad composed by optic nerve hypoplasia, multiple hypothalamic pituitary deficits, and midline brain defects. The midline brain developmental insult in this congenital disorder starts early (between the fifth and eighth gestational weeks) damaging the arrival of GnRH neurons in the hypothalamus that normally occurs by week 13. Pathogenic variants in the genes *HESX1, SOX2, SOX3,* and *OTX2* that encode essential factors for normal forebrain and pituitary development were identified in patients with septo-optic dysplasia (134). Regarding pubertal development, patients with this dysplasia can more

frequently have delayed puberty. However, CPP has been described in 7% of a cohort of 171 patients with septo-optic dysplasia (135). A potential mechanism to explain the premature HPG activation includes the abnormal hypothalamic-pituitary anatomy that may alter the normal suppression of GnRH neurons, leading to earlier onset of pituitary gonadotropin secretion (135, 136).

Tuberous sclerosis complex (TSC) is a relatively rare autosomal dominant neurocutaneous disorder secondary to mutations in the *TSC1* or *TSC2* tumor suppressor genes, which code for the tumor suppressor proteins hamartin and tuberin, respectively (137). The classic triad of this complex disease includes seizures, intellectual disability, and facial angiofibroma. In addition, patients with TSC are at risk of developing multiple benign and malignant tumors in various organ systems (skin, brain/nervous system, kidneys, heart, and lung), resulting in increased morbidity and mortality. Tuberous sclerosis is exceptionally revealed by CPP, and rare cases have been described associated with CNS tumors, such as giant cell astrocytoma or hypothalamic hamartoma and periventricular calcified lesions (138, 139).

Duplication of the pituitary gland is an extremely rare developmental anomaly (140). It may be associated with other midline malformations such as facial anomalies (median cleft lip, median cleft face syndromes, and hypertelorism), vertebral malformations, nasopharyngeal teratoma, and other CNS abnormalities, such as the agenesis of the corpus callosum, posterior fossa abnormalities, the absence of the olfactory bulbs and tracts, the absence of the anterior commissure, and anatomic variations of the circle of Willis. When all these anomalies are present, they are called duplication of pituitary gland-plus syndrome. The hypothalamus and pituitary involvement may be clinically associated with pubertal disorders, such as precocious or delayed puberty (141). The exact mechanism responsible for the early increase in frequency and amplitude of GnRH pulses causing precocious puberty in these patients is still unknown. Burke et al (142) proposed that the same development disorder leading to pituitary duplication may also contribute to the precocious secretion of GnRH from nuclear derangement and failure of regulation of GnRH neurons.

Congenital Causes Without CNS lesions

Gain-of-function Mutations in the Genes Encoding Kisspeptins (*KISS1-KISS1R*)

The important role of the kisspeptins system in GnRH regulation and the identification of mutations in *KISS1* and *KISS1R* associated with congenital hypogonadotropic hypogonadism encouraged researchers to investigate if activation of this system would result in CPP. However, only 1 rare variant in *KISS1* and 1 in *KISS1R* have been reported in patients with CPP. The heterozygous activating mutation of KISS1R (p.Arg386Pro) was identified in association with CPP in 2008 (143). This mutation was identified in an adopted girl who had progressive thelarche from birth, suggesting early, persistent, and slightly increased estrogen secretion. Accelerated growth, skeletal maturation, and progression of breast development were noted at age 7 years. In vitro, the p.Arg386Pro mutation, located in the C-terminal tail of the receptor, led to prolonged activation of intracellular signaling pathways by kisspeptins, resulting in higher and more sustained inositol

phosphate accumulation from decreasing KISS1R degradation (143, 144). Subsequently, 1 rare kisspeptins variant, p.Pro74Ser, was identified in the heterozygous state in a boy who developed sporadic CPP at age 1 year, with high concentrations of basal LH and testosterone (145). In vitro studies showed the capacity to stimulate signal transduction was significantly greater for p.Pro74Ser than for the wild type, suggesting that this variant might be more resistant to degradation, resulting in greater kisspeptin bioavailability (145). His mother and maternal grandmother, both of whom had normal pubertal development, also carried the p.Pro74Ser mutation in the heterozygous state, suggesting incomplete, sex-dependent penetrance. Rhie et al (146) identified polymorphisms that were more frequently seen in patients with CPP and large populational analysis (147) identified a variant close to *KISS1* associated with age at menarche in the normal population without CPP but the association of these variants with pubertal onset still needs to be validated. Although mutations in *KISS1* and *KISS1R* are rarely associated with CPP, this system plays a crucial role in GnRH regulation and has a great potential for target therapies.

Loss-of-function Mutations in Makorin Ring Finger 3 (*MKRN3*)

Despite years of research trying to identify a genetic cause of CPP using a candidate gene approach, only rare mutations had been identified (143, 145, 148, 149). In 2013, an unbiased approach using exome sequencing analysis in multiple families with CPP identified the association of mutations in *MKRN3* with familial CPP (14). In this first report of mutations of *MKRN3*, 4 deleterious mutations—3 frameshift and 1 missense—were detected in 5 of 15 families (33%) with several members with CPP. After this first report of *MKRN3* mutations in familial cases of CPP, mutations in *MKRN3* were also identified in patients without a known family history of CPP (150). *MKRN3* mutations are now the most commonly known genetic defect associated with CPP, with an overall frequency of ~10%; the frequency of mutations in *MKRN3* is higher in cases of familial CPP, ~33% to 46% (14, 151, 152).

MKRN3 is a member of the Makorin protein family (153, 154). The MKRN3 protein has a centrally located RING finger motif (C3HC4), 2 amino-terminal C3H zinc finger motifs followed by a Makorin zinc finger motif unique to the Makorin protein family, and a carboxy-terminal C3H zinc finger motif (Fig. 3) (153). C3H zinc-finger motifs have been implicated in mRNA binding, whereas the RING zinc-finger motif is responsible for E3 ubiquitin ligase activity (155). These several domains suggest that MKRN3 has multiple actions. MKRN3 is highly conserved among species, and the mouse and human MKRN3 amino acid sequences share 69% identity and 82% similarity (153). Mice and humans usually do not have conserved UTRs, yet the *MKRN3* 3′-UTR has 90% identity between these 2 species, suggesting a functional significance to this region of the *MKRN3* gene (153). Indeed, the conserved sequence includes 2 ATTTA motifs, a binding site for miR-30, which is a repressor microRNA. miR-30 is expressed in KNDy neurons and is the first element shown to regulate *MKRN3* expression (156).

MKRN3 also interacts with and suppresses the activity of Nptx1, a secreted protein important for neuron development (158). The RING finger domain of MKRN3 is essential for binding with and for polyubiquitination of Nptx1 during
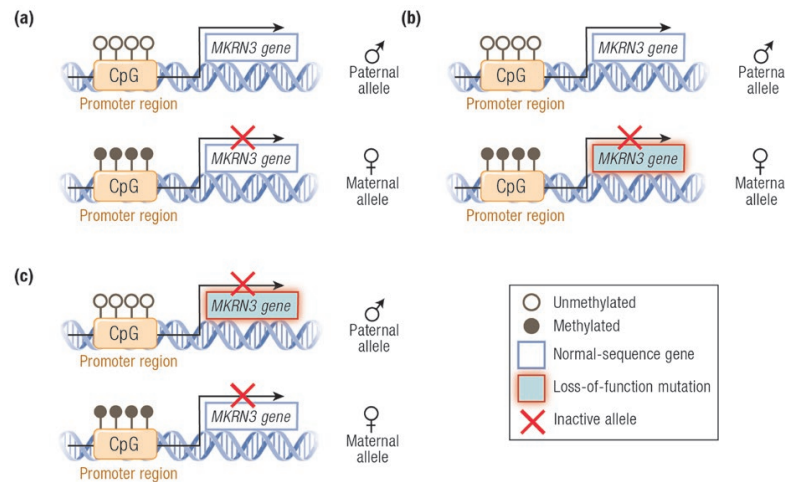
Figure 3. Representation of the 2 autosomal alleles of the imprinted *MKRN3* gene and distinct genotypes of affected and unaffected CPP individuals. (A) Normal pattern of imprinting of *MKRN3* gene, with silencing of the maternal allele (by methylation of its promoter region) and monoallelic expression of the paternal allele. (B) The genotype of an individual who inherited a maternal loss-of-function mutation. The individual will not present with CPP because the paternal allele is normally expressed. (C) The genotype of an individual who inherited a paternal loss-of-function mutation. The individual will present with CPP because both alleles are inactive: the paternal is mutated and the maternal is silenced. Adapted from Canton et al (157).

puberty initiation (158). Missense mutations in *MKRN3* identified in patients with CPP, encoding p.Cys340Gly and p.Arg365Ser, located in the RING finger domain, and p.Phe417Ile and p.His420Gln, located in the C-terminal zinc finger domain, resulted in loss of the ability to inhibit *GnRH1* promoter activity (68). The 2 RING finger MKRN3 mutants also lost the ability to inhibit *KISS1* and *TAC3* promoter activity (64). These data not only confirm that these mutations result in loss of function of MKRN3 but also implicate the RING finger ubiquitin ligase domain as an important functional domain of MKRN3.

MKRN3 mutations in association with CPP have been described in more than 150 patients from many countries (13–15, 86, 150, 152, 159–176). However, *MKRN3* mutations were not identified in a subgroup of 260 Korean girls with familial CPP (172) and a lower frequency of mutations in familial cases was also described in other series from Italy and Turkey (8.7% and 5.3%, respectively) (170, 176).

MKRN3 is located on the long arm of chromosome 15, in a region with a cluster of imprinted genes. Some of these genes are expressed only from the paternally inherited allele and are associated with Prader-Willi syndrome (PWS), whereas others are only maternally expressed and associated with Angelman syndrome (153, 154). *MKRN3* is included in the cluster of genes with exclusive paternal allele expression. The schematic representation of the 2 autosomal alleles of the imprinted *MKRN3* gene and distinct genotypes of affected and nonaffected CPP individuals is shown in Fig. 3 (157). Studies have shown that *MKRN3* deletion is neither required nor responsible for the PWS phenotype, with several cases of PWS presenting without deletion of *MKRN3* (177–179). Moreover, the inclusion of *MKRN3* within the deletion causing PWS does not predict the pubertal phenotype because most patients with PWS with deletions including *MKRN3* do

not develop CPP (177, 180, 181). Kanber et al (178) reported a patient presenting with signs of PWS—obesity, high pain threshold, and developmental delay—with an unbalanced translocation resulting in the deletion of *MKRN3*, *MAGEL2*, and *NDN* genes. After further phenotypic evaluation, the patient was determined to have CPP but not PWS. To date, at least 16 additional cases have been reported with PWS and CPP (182–184). The early pubertal onset in these patients is likely from loss of *MKRN3*. It is important to highlight that the CPP phenotype may not be documented in patients with PWS, who have other clinical features that can obscure detection of early pubertal development, especially in boys given the subtlety of the first signs of pubertal onset. It is also worth noting that hypogonadism, either primary or secondary, is a major feature of PWS. Even with early reactivation of GnRH secretion, the lack of precocious pubertal development in these patients may be due to the associated inability to secrete gonadotropins or sex steroids due to concomitant hypogonadism.

To date, 59 inactivating mutations in the coding sequence of *MKRN3* have been described, including 6 nonsense, 16 frameshift, and 37 missense mutations (Fig. 4). Missense mutations account for 63% of *MKRN3* mutations, frameshift for 27%, and nonsense for 10%. Of the 59 mutations, 15 were found within sequences encoding the 3 C3H1 zinc finger motifs, which have RNA binding activity, and 2 mutations were found in the Makorin type zinc finger domain. Twelve mutations were found in the RING finger C3HC4 domain, responsible for E3 ubiquitin ligase activity. Of these, 10 were missense mutations. Thirty (51%) mutations were outside of these domains: 11 were frameshift, 3 nonsense, and 16 missense mutations. Notably, the 11 frameshift mutations occurred between the first 2 C3H1 domains, highlighting an area susceptible to pathogenic frameshift mutations.
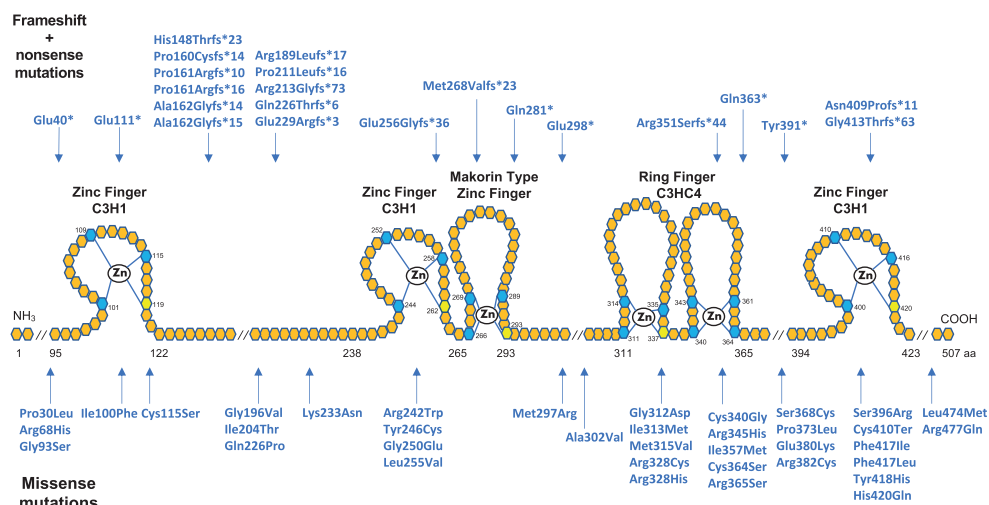
Figure 4. Schematic representation of MKRN3 protein structure and location of loss-of-function mutations identified in patients with CPP. Hexagons represent individual amino acids, and corresponding numbers indicate amino acid positions. Top row mutations are frameshift and nonsense, whereas bottom row are missense mutations. Blue and yellow hexagons represent key cysteine and histidine amino acids, respectively, necessary for zinc ion interaction. RING finger C3HC4 is a protein-binding domain responsible for ubiquitin ligase activity. Zinc finger C3H1 are RNA binding domains. Makorin type Zinc finger is a specific Cys–His domain identified in the proteins of the makorin family. Notably, 15 mutations (27%) were detected between the first 2 C3H1 domains, 11 of which are frameshift. Mutations also tend to cluster within the C3HC4 RING finger domain (20%), the vast majority of which are missense.

The most prevalent *MKRN3* mutation, identified in at least 34 patients, is an indel variant affecting a poly-C region at cDNA positions 475-481 (7 cytosines), which results in a frameshift mutation with a premature stop codon (150, 161). The resulting amino acid change is either at Pro160, Pro161, or Ala162 (159). Figure 4 shows the varying nomenclatures describing the mutations reported in this hotspot region (159).

Two unrelated girls with nonsyndromic CPP were found to have heterozygous whole gene deletion of *MKRN3*. One had a 584-kb deletion (GRCh37/hg19 chr 15:23 798 088-24 382 443) at 15q11.2 involving *MKRN3*, *MAGEL2*, and *NDN* genes (185). Consistent with the imprinted and paternal expression of *MKRN3*, both deletions were confirmed to be paternally inherited. The 2 patients had no family history of CPP and had no symptoms suggestive of PWS. One patient had a BMI in the 75th percentile; the other patient, with a larger deletion, had a BMI in the 99th percentile for her age and both had a family history of obesity. Given the prevalence of obesity, it is difficult to draw conclusions about the relevance of the deletion to this phenotype. An increase in obesity in patients with nonsyndromic CPP from *MKRN3* mutations has not been detected, compared with those with CPP without *MKRN3* mutations (151, 159). Premature ovarian failure was reported in the grandmother of an individual with CPP harboring an *MKRN3* mutation (168). Additional dysmorphisms were rare and included esotropia detected in 2 affected siblings, and clinodactyly and lumbar hyper lordosis in 2 unrelated girls, one of whom also exhibited a high-arched palate and dental abnormalities (150, 168). All these findings could have been incidental.

Patients with CPP carrying loss-of-function mutations in *MKRN3* exhibited typical clinical and hormonal features of premature activation of the HPG axis, including early pubertal signs, such as breast development, testis enlargement, accelerated linear growth, advanced bone age, and elevated basal and/or GnRH-stimulated LH levels. Most studies reported no significant differences in clinical and laboratory features of patients with CPP with or without *MKRN3* mutation (151, 186). However, Simon et al (152) described that girls with *MKRN3* mutations were younger at puberty onset than those without *MKRN3* mutations. Seraphim et al (159) showed an earlier age at diagnosis in female patients with CPP associated with *MKRN3* mutations, when compared with girls with idiopathic CPP. The earlier identification of pubertal onset likely resulted from family awareness of the diagnosis of CPP because of the higher frequency of familial cases in the cohort (159). Because CPP is significantly more frequent in girls, the number of boys with *MKRN3* mutations is smaller, making it difficult to draw associations. Bessa et al (86) showed a higher frequency of *MKRN3* mutations in boys with CPP and no CNS lesions than in girls. It has been proposed that boys have a smaller advance in the timing of puberty onset compared with girls (173, 176). However, this may be due to difficulties in identifying testicular enlargement, the first clinical evident sign of pubertal onset in boys. In fact, most boys included in these studies were diagnosed based on family history, many later in their pubertal development, highlighting the challenges of diagnosis of CPP in boys. Two studies with large patient sample sizes described higher basal FSH levels at the time of the diagnosis in girls with CPP with *MKRN3* mutations compared with CPP girls without *MKRN3* mutations (150, 159). It is unclear why higher FSH

levels were found in patients with CPP and *MKRN3* mutations; the difference might be attributable to the impact of different frequencies of pulsatile GnRH release on gonadotropin secretion (150).

In a study by Seraphim et al (159), significantly greater bone age advancement and higher basal LH levels were found in patients harboring frameshift, stop codon, and promoter region mutations in *MKRN3*, compared with those harboring missense *MKRN3* mutations. The response to GnRH analogue (leuprolide acetate) treatment in patients with CPP with *MKRN3* mutations was assessed in 11 girls treated for a mean time of 2.9 years. These girls reached expected family height similarly to girls with CPP without *MKRN3* mutations, demonstrating the efficacy of GnRH analogues in preserving genetic adult height potential, regardless of the etiology of CPP (186). This study also showed that the prevalence of metabolic and reproductive disorders was similar in patients with CPP because of *MKRN3* mutations compared to those with idiopathic CPP.

In summary, *MKRN3* is the first gene with loss-of-function mutations identified in humans with an inhibitory effect on GnRH secretion. It is also the first imprinted gene associated with non-syndromic CPP.

Loss-of-function Mutations in *DLK1*

DLK1, also known as preadipocyte factor 1 or fetal antigen 1 is a key element of the Notch signaling pathway and several other intracellular pathways. It is widely expressed in different tissues during embryonic development, including adipocytes and muscular tissue; however, at postnatal life in humans, the expression is highest in endocrine glands, mainly in adrenals, pituitary, pancreas, and gonads (187).

In 2017, Dauber et al (63) described a complex defect of *DLK1* (~14-kb deletion encompassing the first exon and 269-bp duplication) in a large multigenerational family with CPP. The *DLK1* mutation was identified in all 5 patients through an innovative approach, including linkage analysis followed by whole-genome sequencing (63). The CPP phenotype was only expressed when the mutant gene was inherited from the father, in an inheritance compatible with the imprinting pattern of *DLK1* (ie, the maternal allele is silenced). It is notable that *DLK1* is located in a cluster of imprinted genes at chromosome 14q32.2, which has been associated with Temple syndrome (188, 189).

Temple syndrome has several distinct clinical features, such as short stature, small hands and feet, truncal obesity, and, interestingly, early onset of puberty, which has been described in 80% to 90% of affected subjects (189, 190). Notably, the deletion of *DLK1* described by Dauber et al (63) led to CPP and a higher prevalence of metabolic syndrome, without the other syndromic features. Confirming the importance of this monogenic cause of CPP, Gomes et al (191) performed *DLK1* sequencing analysis in 60 patients with either CPP or a history of precocious menarche. Three distinct heterozygous frameshift mutations in exon 5 of *DLK1* (p.Gly199Alafs*11, p.Val271Cysfs*14, and p.Pro160Leufs*50) were identified in 5 patients from 3 unrelated families. The metabolic phenotype became more evident as the number of affected women with DLK1 deficiency increased: overall the group had more obesity/overweight (60%), insulin resistance (70%), type 2 diabetes (30%), and high cholesterol levels (50%) when compared with a paired idiopathic CPP cohort of 20 females (191). It is noteworthy that the extracellular soluble portion of DLK1 can be quantified in the serum, using a commercially available soluble DLK1 ELISA. In both studies, serum DLK1 levels were measured in the affected subjects and in
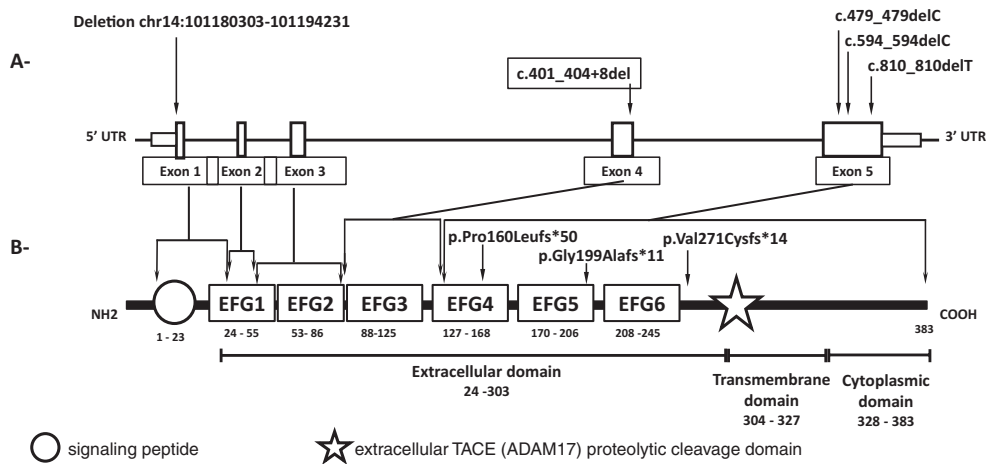
Figure 5. Schematic representation of the human DLK1 gene (A) and protein (B), respectively. (A) Human *DLK1* gene (transcript ENST00000341267.9): clear blue boxes indicate the coding sequences (5 exons) of the gene. The localization of the allelic variants identified in familial CPP (63, 191) are indicated by arrows.(B) Human DLK1 protein structure (P80370): the orange circle indicates the signaling peptide; dark blue boxes indicate the six EGF-like repeats. Blue star indicates the extracellular TACE (ADAM17) proteolytic cleavage domain. Purple boxes indicate the region of the protein affected by the mutation in DLK1. The numbers represent the amino acid positions of the indicated domain. EGF: epidermal growth fator. Adapted from Montenegro et al. (192).

controls, and were undetectable in all affected subjects, confirming the loss of gene function (63, 191). In 2020, a collaborative study between Brazilian and Spanish centers identified a novel heterozygous de novo deletion in exon 4 of *DLK1* (c.401_404 + 8del) in a girl with sporadic CPP who had undetectable serum DLK1 levels (192). The schematic representation of the human DLK1 gene and protein is shown in Fig. 5.

Notch signaling is a highly preserved signaling pathway that consists in the interaction of a notch receptor and a Notch ligand (193). In mammals there are 4 types of notch receptors (ie, NOTCH1, NOTCH2, NOTCH3, and NOTCH4), all of which have a single transmembrane passage. When the extracellular domain interacts with a notch ligand from another cell (ie, *trans* interaction), a series of enzymatic cleavages (ADAM/TACE and γ-secretase) results in the release of the intracellular domain, which acts in the nucleus of the receiving cell (the cell with the NOTCH receptor). The canonical ligands are characterized by a DSL domain (delta, serrate, and lag2); however, a large number of noncanonical ligands exist, among which is the DLK1 (193). Although most ligands elicit the series of activating events previously described, DLK1 has an inhibitory role on notch signaling. It usually interacts with NOTCH receptors in "cis" (that is, the receptor and the DLK1 are in the same cell, and not in different cells) or through its cleaved extracellular domain in an almost paracrine way. These types of interaction do not generate the force necessary to expose the NOTCH receptor extracellular domain to cleavage. Therefore, the DLK1 represents an inhibitor of Notch signaling (159).

Beyond the identification of loss-of-function mutations in *DLK1* in patients with familial CPP, further evidence has pointed to a role of DLK1 in the regulation of pubertal timing. First, genome-wide association studies had implicated *DLK1* as 1 of the 3 imprinted *loci* associated with the age at menarche in a large cohort of healthy women of European ancestry (147). Second, albeit a widespread expression during embryonic development, postnatal *Dlk1* mRNA and protein expression is restricted to some endocrine tissues such as the pituitary, adrenal gland, pancreas, testes, prostate, ovaries, and a subset of neurons in the central nervous system (194). Immunohistochemistry in the mouse hypothalamus revealed neuronal Dlk1 expression in the suprachiasmatic, supraoptic, paraventricular, lateral, dorsomedial, and arcuate nuclei (195). Indeed, Dlk1- producing neurons are particularly abundant in the hypothalamic arcuate nucleus, a main site for the neuroendocrine control of puberty, especially for harboring kisspeptin neurons (196). Moreover, Dauber et al (63) demonstrated *Dlk1* mRNA expression in immortalized AVPV- and arcuate-specific neuronal kisspeptins cell lines. Third, 2 *Dlk1* mRNA isoforms have been described in the hypothalamus: 1 translated into the full-length protein and a second form that produces a shorter protein of 30 kDa composed only of the extracellular domain of Dlk1 (195). Interestingly, the 30-kDa soluble and biologically active form predominates in the hypothalamus (195). In addition, Dlk1 has been shown to act as an inhibitor of Notch signaling, and Rbpjκ-dependent Notch signaling regulates progenitor maintenance and differentiation of hypothalamic arcuate neurons (197). Biehl et al (198) have shown that manipulating Rbpjκ-dependent Notch signaling affects kisspeptin neuronal development. Although the precise mechanisms by which Dlk1 blocks puberty remain unknown, taken together these data

advocate for a potential function of Dlk1 on the regulation of the reproductive axis (198).

It is well documented that pubertal activation of the reproductive axis and maintenance of fertility are critically dependent on the body's energy reserves and metabolic status (199). DLK1 has been implicated in several aspects of energy metabolism, including its function as an inhibitor of adipogenesis by preventing the preadipocyte proliferation and differentiation into mature adipocytes (200) and its association with insulin resistance in both rodents and humans (201–203). The presence of DLK1 protein in most orexigenic Agouti-related protein and NPY neurons and only to a lesser extent in anorexigenic cocaine- and amphetamine-regulated transcript containing neurons and the *DLK1* mRNA regulation by nutritional challenges strengthen its role in hypothalamic control of body weight, possibly through actions on the development and synaptic plasticity of neurons in the arcuate (196, 204). In addition, DLK1 is involved in beige fat biogenesis and adaptive thermogenesis (205). Moreover, Wermter et al (206) analyzed a large cohort of trio families (2 parents and 1 obese child) from European descent and identified a synonymous polymorphism within the *DLK1* gene in association with childhood and adolescent obesity.

Likewise, *Dlk1*-deficient mice have an accelerated weight gain that occurs at later ages and is associated with fat deposition, dyslipidemia, and enlarged fatty liver (207). Conversely, mice overexpressing *Dlk1* were shown to have limited accumulation of adipose tissue and reduced liver steatosis during postnatal life, even when challenged with a high-fat diet (208). Intriguingly, more recent data from humans exhibited a positive correlation of DLK1 circulating levels with BMI, fat content, and insulin resistance (200, 209). In agreement with these findings, *Dlk1*-deficient mice exhibit protection against insulin resistance and obesity induced by a high-fat diet. These results parallel the data regarding leptin in humans, whereas congenital leptin deficiency leads to early-onset severe obesity, patients with acquired obesity have higher levels of leptin because of leptin resistance (210). Either way, acting as a gatekeeper or as an enhancer of adipogenesis in different contexts, the evidence so far has unveiled the importance of Dlk1 for this differentiation process and suggest a possible role of Dlk1 as a metabolic regulator of reproduction.

Syndromic CPP Without CNS Lesions

CPP without CNS lesions is mostly frequently described as an isolated entity, but it may also present combined with other signs and symptoms, encompassing a syndromic form (16). To date, few studies have contributed to identifying patients with syndromic disorders among large CPP cohorts (16, 211). In this setting, a promising translational study investigated 36 selected patients with CPP associated with multiple anomalies through (epi)genetic studies (16). Rare genetic abnormalities were identified in 12 (33%) of them, including genetic defects in *loci* known to be involved with CPP (14q32.2 and 7q11.23; further discussed in a later section) or candidate chromosomal regions or genes.

CPP has been demonstrated to be part of the phenotypic spectrum of rare genetic syndromes caused by distinct defined molecular mechanisms, such as epigenetic defects, CNVs, and gene mutations (16, 211). Recently, the premature activation of the reproductive axis has been described

as a possible component of imprinting disorders, a group of congenital diseases caused by disturbances in imprinted genes affecting growth, development, and metabolism (212). Temple syndrome (OMIM 616222) is a rare imprinting disorder marked by precocious puberty in 80% to 90% of cases. Additionally, patients characteristically present with prenatal and postnatal growth failure, hypotonia, small hands and/or feet, and obesity (16, 189). It is caused by the disruption of the chromosome 14q32.2, a chromosomal region carrying a cluster of imprinted genes, including *DLK1* and its primary imprinting control center, the *DLK1/MEG3*:intergenic-differentially methylated region (*DLK1/MEG3*:IG-DMR) (213). Three main 14q32.2 molecular abnormalities can underlie Temple syndrome phenotype: maternal uniparental disomy of chromosome 14, hypomethylation of the *DLK1/MEG3*:IG-DMR on the paternal allele (epimutation), and paternal deletion of the *DLK1/MEG3* domain (189, 213). These 3 mechanisms harbor in common the lack of expression of the paternal copy of *DLK1* gene and clinically may manifest CPP (16, 189, 213). Interestingly, barely detectable levels of serum DLK1 were measured in Temple syndrome patients from epimutations and deletions at chromosome 14q32.2 (212). Based on these lines of evidence, it has been postulated that *DLK1* deficiency is probably the leading cause of premature pubertal development in Temple syndrome patients (16).

PWS (OMIM 176270) is a classic imprinting disorder, mostly characterized by hypotonia, obesity, growth failure, cognitive disabilities, and hypogonadism, but has also been associated with precocious puberty albeit infrequently (about 4%) (179). PWS occurs from an absence of the paternally expressed imprinted genes at chromosome 15q11-q13. The *MKRN3* gene, the most prevalent factor associated with familial CPP, is located at the boundary of this critical region (179). Meader et al (214) investigated paternal deletions in this region and they postulated that the low frequency of early puberty in Prader-Willi syndrome patients was most likely from concomitant disturbances leading to hypogonadism (214).

Silver-Russell syndrome (OMIM 180860) is a clinically and (epi)genetically heterogeneous imprinting disorder, mainly characterized by prenatal and postnatal growth retardation. The most common mechanisms are hypomethylation of the *IGF2/H19*:IG-DMR and maternal uniparental disomy of chromosome 7 (UPD(7)mat) (215). Interestingly, precocious puberty was described in some of the first cases reported in the historical cohorts of Silver-Russell syndrome (216). More frequently, patients develop early puberty, characterized by age at puberty onset at the younger limit of the normal range (212, 215). Children with UPD(7)mat are likely to develop puberty at younger ages (215), possibly from still unknown pubertal influencing factors located in this chromosome. Interestingly, another well-known chromosome 7 abnormality (Williams-Beuren syndrome) can present with premature sexual development. Williams-Beuren syndrome (also called Williams syndrome; OMIM 194050) is a multisystem disorder caused by hemizygous 7q11.23 deletion, leading to a contiguous gene syndrome with clinical heterogeneity (217, 218). The main clinical features are distinct craniofacial appearance, cardiovascular disease, short stature, intellectual disability, and hypersociability (218). Affected children may present an early puberty (up to 50%) or CPP (3%-8%) (217–219). Syndromic girls were reported to have menarche approximately 2 years earlier than control girls (217, 219). Putative genotype-phenotype correlations for genes within

the 7q11.23 deletion have been described (218). However, the exact gene or mechanism involved in this premature puberty phenotype remains unknown. Remarkably, an enriched signal for association with age at menarche within the critical region of Williams syndrome was identified in a large genome-wide association study, increasing the likelihood for this chromosome to carry a potential pubertal influencing factor (12).

Different studies have associated CPP with other rare CNVs, such as 1p36 deletion (220), 9p distal deletion (221), and 9q34.3 duplication (including *NOTCH1* gene) (222). Two distinct CNVs syndromes involving the X chromosome were shown in association with a premature puberty phenotype (16, 223). Canton et al (16) identified three familial CPP cases carrying Xp22.33 deletions, involving the pseudoautosomal region 1 (*SHOX* gene region). The pedigree analysis revealed that early puberty cosegregated with *SHOX* phenotypes (body disproportion, short stature, and Madelung deformity) in a dominant pattern of inheritance. The exact mechanism involved in the pubertal phenotype of these cases remains to be elucidated (16).

The rare Xp11.23-p.11.22 duplication syndrome (OMIM 300881) is a contiguous gene syndrome with an X-linked dominant inheritance, mainly characterized by intellectual disability, speech delay, electroencephalogram abnormalities, overweight, skeletal anomalies, and early puberty (223). Since the first report by Giorda et al (223), precocious pubertal development has been described in familial and sporadic cases. Evidence from 3 studies showed precocious puberty in around 11% of males and 70% of females, the latter presenting age at menarche from 8 to 9 years (223–225).

The putative role of genes on the X chromosome in the genetic architecture of human pubertal timing may also be suspected by the description of rare cases of defects in the X-linked gene methyl-CpG-binding protein 2 (*MECP2*) presenting with precocious puberty (226–228). Loss-of-function mutations in *MECP2* are associated with neurodevelopmental disorders, mainly with Rett syndrome (OMIM 312750), an X-linked dominant disorder occurring mostly in females (226). Remarkably, CPP has been described in atypical or rare cases of girls with Rett syndrome (226, 227). A boy with severe intellectual disability and CPP was described with an Xq28 duplication including *MECP2* (228). Interestingly, precocious puberty was also described in up to 13% of girls with dominant de novo mutations in the X-linked gene dead-box helicase 3 X-linked (chromosome Xp11.4), which causes an X-linked intellectual development disorder (OMIM 300958) (229).

Autism spectrum disorder is also a CNS disorder that has been associated with X-linked genes defects and sporadically with CPP (229, 230). A cohort study of health care data from Florida (USA) compared children with or without autism spectrum disorder. Notably, the autism group had a higher risk of CPP (adjusted hazard ratio, 4.64) (230).

Epigenetics Role in CPP

Epigenetics is defined as the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence. The epigenetic marks encompass maintained chemical additions to DNA and histones that play a key role in regulating chromatin and/or gene expression and lead to gene silencing or activation (231). Epigenetic information in mammals can be transmitted by the following main mechanisms: mitotically stable DNA

methylation; posttranslational modifications of histone proteins; chromatin remodeling and noncoding RNAs (such as miRNAs and long noncoding RNAs) (232, 233). The loss of effectiveness of epigenetic regulation has been associated with distinct human diseases (122, 233).

In the past decade, growing evidence has demonstrated the participation of epigenetic mechanisms in the control of puberty, indicating its potential modulating role in human pubertal timing (12, 14). Indeed, the identification of disease-causing inactivating mutations in imprinted genes in several families with CPP has suggested a role for epigenetic mechanisms in this hypothalamic disorder (14, 63). Since the first report by Abreu et al (14), inactivating mutations in the imprinted gene *MKRN3* have been described as the most common cause of monogenic CPP. More recently, loss-of-function mutations in the *DLK1* gene were described as a rarer cause of CPP (63, 191). Interestingly, both genes are paternally expressed from the imprinting of the maternal allele, leading to its silencing (see sections on *MKRN3* and *DLK1* mutations). Genomic imprinting is an epigenetic process of gene silencing established through DNA methylation of promoter or regulatory regions by which selected genes are expressed in a parental-specific manner, leading to their monoallelic expression (233). Based on this, Canton et al (16) investigated imprinting defects of *MKRN3* and *DLK1* through methylation-specific analysis in a subset of patients with idiopathic CPP associated with multiple anomalies (n = 36). Three patients were identified with hypomethylation at the *DLK1* imprinting control region, an epigenetic mechanism associated with Temple syndrome, which may be clinically subtle among CPP cohorts (see section on syndromic CPP). No DNA methylation defects were identified in the *MKRN3* promoter region (16). Similarly, in an Asian study, no abnormality of *MKRN3* methylation was detected in 19 patients with CPP (234). These data suggested that *MKRN3* methylation defects are not a common cause of CPP, whereas *DLK1* hypomethylation may be considered in patients with sporadic CPP resembling Temple syndrome.

Genome-wide DNA methylation patterns associated with pubertal timing were investigated in CPP and healthy girls. Bessa et al (235) evaluated the methylome profiling in leukocytes of 10 CPP girls and 33 healthy prepubertal and pubertal girls. A widespread pattern of DNA hypermethylation was identified in girls with both normal and abnormal puberty, indicating that the pubertal process in humans is associated with specific changes in epigenetically driven regulatory control. There was an enrichment of changes in methylation of zinc-finger genes, including hypomethylation at the promoter of *ZFP57* in human peripheral leukocytes and, also in the hypothalamic region of female rhesus. This elevated hypothalamic *ZFP57* expression during peripubertal development indicated potential enhanced repression of downstream *ZFP57* target genes (235).

In a study of 34 healthy American girls, a hypermethylation pattern in leukocyte DNA was demonstrated during pubertal development (236). In addition, 3 more studies analyzed longitudinally genome-wide changes in leukocyte DNA methylation of healthy girls and boys during pubertal development, identifying several differentially methylated genomic regions related to normal puberty (237–239). Almstrup et al (237) identified the most significant region for both sexes in the promoter region of Thyroid Hormone Receptor Interactor 6 (*TRIP6*), located at 7q22. Measurements of circulating levels of TRIP6 in healthy pre-, mid-, and postpubertal healthy Danish children evidenced a significant increase as individuals progressed through puberty. Therefore, the methylation of the promoter of *TRIP6* was potentially associated with pubertal development (237). However, little is known about the functional role of human *TRIP6*.

Several important animal studies have corroborated the emerging role of epigenetic mechanisms in pubertal timing (240). There is an emerging concept that a switch from epigenetic repression to activation within arcuate kisspeptin neurons is a core mechanism underlying the initiation of puberty (232). Lomniczi et al (241) identified an epigenetic mechanism of transcriptional repression that regulated the initiation of female puberty in rats. The principal contributors to this mechanism of repression were silencers of the Polycomb group (PcG) of proteins, which could affect the gene network that is involved in the stimulatory control of puberty, including *Kiss1* and glutamatergic receptors genes (242). The kdm6b enzyme was identified as a central node of this network by erasing the PcG-dependent histone modification of H3K27me3. It was suggested that Kdm6b repression could be a PcG mechanism to modulate puberty-activating gene networks (232, 242). Zinc-finger factors have also been implicated as an epigenetic modulator of puberty through processes of transcriptional repression (243). In studies using monkeys and rodents, *GATAD1*, a zinc finger protein decreasing in peripuberty, was identified as a key player for the repression of *Kiss1* and *Tac3* in the arcuate neurons and restraining puberty (243). The Trithorax group (TrxG) of proteins is a complex of transcriptional activators (232). Notably, both PcG and TrxG are chromatin proteins known to have mutually antagonistic activity. TrxG proteins are known to antagonize PcG proteins by implementing methylation of histone marks at H3K4 (232). Animal studies evaluating interactions with the *Kiss1* promoter have shown that, before initiation of puberty, as the PcG-mediated gene silencing declines, the TrxG activation increases, configuring a fine-tuned dual process of epigenetic regulation of pubertal timing (232).

A study in mice evidenced that a miRNA switch could regulate the rise in hypothalamic GnRH production before puberty, with miR-200 and miR-155 being identified as 2 essential components of this process, regulating transcriptional factors of *GnRH*, and interfering in the correct initiation of puberty (244). Subsequently, an experimental study in rats demonstrated an increase in hypothalamic miR30-b expression during postnatal maturation that was associated with a concomitant *Mkrn3* decrease. Functional in vitro analyses demonstrated a strong repressive action of miR-30b on *Mkrn3* 3′UTR and consequently reduction of *Mkrn3* expression (156). Interestingly, circulating relative miR30-b levels was assessed in 26 boys with constitutional delay of growth and puberty (245). The miR30-b levels increased during puberty in these boys, appearing to be related to the HPG axis activity (245). Changes in mir30-b and other miRNAs causing CPP in humans are still to be unmasked.

Acquired Etiology of CPP

Acquired Causes With CNS Lesions

Different acquired conditions affecting the hypothalamus or brain, such as tumors, cranial irradiation, infection, trauma, and hydrocephalus, have been recognized as classical causes

of premature GnRH secretion and, therefore, CPP (3, 9). However, the precise mechanism is not completely understood in the great majority of these conditions. The mechanisms by which distinct neoplastic or nonneoplastic lesions result in CPP remain hypothetical (246). Most evidence suggests that tumor sites near the hypothalamic-pituitary region disrupt the neuroendocrine balance with consequent early secretion of GnRH (9, 246). Increased intracranial pressure represents another possible explanation of organic CPP (246).

The main risk factors for CPP from CNS abnormalities include a younger age and male sex (246). Chalumeau et al (247) recognized 3 predictors of CNS lesions in girls, including age <6 years, elevated basal estradiol levels (>110 pmol/L), and absence of pubic hair.

Tumoral Causes

The presence of CNS tumors in CPP is relatively uncommon (248). In a systematic review including 15 studies and 1853 girls with CPP, the incidence of CNS tumors was 1.6% (80). These tumors included astrocytomas, gliomas, craniopharyngiomas, suprasellar tumors, hypothalamic tumors, pontine tumor, pinealoma, and fourth ventricular tumor (80). A recent study involving brain MRI of 770 girls with CPP revealed 13.5% with CNS lesions, with only 2 tumors (0.25%) being identified, including 1 low-grade glioma and 1 meningioma (89). The clinical presentation of CNS tumors is related to the tumor expansion velocity, its location, and the age of the child. Clinical findings can be quite varied, ranging from signs of increased intracranial pressure to localizing neurological signs and symptoms. Infratentorial tumors can occur in the posterior fossa (medulloblastomas, cerebellar astrocytomas, gliomas, and ependymomas) and their clinical presentations consist in signs of increased intracranial pressure, headache, emesis, weight loss, ataxia, and neuropathy. If CPP presents in this setting, it is most likely secondary to increased intracranial pressure causing interference in the hypothalamic region. Supratentorial tumors (craniopharyngiomas, gliomas, germinomas, pineal tumors, ependymomas) manifest with visual disturbances, signs of increased intracranial pressure, macrocephaly, and neuroendocrine dysfunction such as growth failure or diabetes insipidus (9).

Low-grade gliomas represent more than 40% of CNS tumors (124). Juvenile pilocytic astrocytoma is the most common lesion type with unpredictable growth and the possibility of spontaneous involution, late-onset progression, or leptomeningeal metastases can occur (124). Ten to 16% are linked to NF1 and although 50% to 60% of pediatric low-grade gliomas involve the cerebellum, cerebral hemispheres or brainstem, 30% to 50% affect the optic nerves, chiasm, and tracts, the hypothalamus, and suprasellar midline (124). Interestingly, precocious puberty has not been described in optic glioma, astrocytoma, or ependymoma localized outside the suprasellar area. As mentioned previously, hypothalamic astrocytes can secrete neuroactive substances that stimulate the release of GnRH (249) and they accelerate GnRH neuronal phenotypic differentiation (250). Therefore, in children with this type of tumor, CPP might be caused by tumor location, and/or tumor cell type (246).

Pineal tumors include germ-cell tumors, pineal parenchymal tumors, and glial tumors. These lesions comprise as much as 7% of CNS tumors in childhood (246, 251). The pineal gland is located near the cerebral aqueduct; therefore,

tumors rising in this location may also produce obstructive hydrocephalus and CPP. In this context, precocious puberty may occur either by tumor-induced hydrocephalus resulting in CPP, or through hCG secretion from germ-cell secreting tumors originating a GnRH-independent or peripheral form of precocious puberty (246). In fact, all tumoral lesions arising in locations potentially related to obstructive hydrocephalus can evolve with CPP.

Other Conditions

Several insults, such as neonatal infections, cerebrovascular accidents, hydrocephalus, traumatic brain injury, cranial irradiation, and encephalopathies, can be associated with CPP (11). An increased pressure on the hypothalamic-pituitary area with loss of normal childhood hypothalamic inhibition of pituitary gonadotropins could be one of the factors responsible for CPP. Cranial radiation largely used for the treatment of primary CNS tumors can result in CPP through direct effects on GnRH secretion (246, 252). Radiation therapy for childhood cancers represents a cause of early reactivation of GnRH secretion (253). Lower doses (18-24 Gray) of radiotherapy were associated with precocious puberty in girls, whereas doses higher than 25 Gray affected both sexes. Notably, younger age at radiotherapy confers a higher risk of CPP (253, 254). This modality of treatment might cause damage to GABAergic neurons, which could lead to premature activation of GnRH neurons (253, 255).

Children affected by neurodevelopmental disability could experience early pubertal changes at least 20 times more frequently than the general population (256). Limited data about CPP among children affected by cerebral palsy are available (257); however, a multicenter, cross-sectional study including 207 children (84 girls and 123 boys aged 3-18 years; 71% White) with cerebral palsy of moderate to severe motor impairment demonstrated that puberty begins earlier but ends later in children with cerebral palsy, compared with children in the general population (256, 257). In addition, menarche occurs later in girls with cerebral palsy, with more advanced sexual maturation being associated with more body fat in girls but less body fat in boys (257). These clinical observations suggest that early activation of the HPG axis in patients with cerebral palsy is due to a loss of hypothalamic inhibition of GnRH secretion during childhood (256, 257). Moreover, severe brain damage and antiepileptic drugs may affect some neurotransmitter pathways involved in gonadotropin control (258). Notably, most patients with cerebral palsy need antiepileptic drugs treatment with a potential interference in pubertal timing.

Acquired Causes of CPP Without CNS Lesions

Endocrine-disrupting Chemicals

EDCs are chemical compounds (natural or synthetic) that may alter endocrine and homeostatic systems by affecting hormone secretion or their targets (259, 260). The sources of substances known as EDCs may include drugs, pesticides, fungicides, plasticizers, industrial products, cosmetics, heavy metals, and naturally botanical chemicals. The comprehension of the mechanisms of action and the consequences of exposure to EDCs must consider key aspects, such as age at exposure, latency from exposure, mixture of compounds, nontraditional dose-response dynamics, and possible epigenetic effects (259).

Nevertheless, several studies have provided evidence of the effects of EDCs on endocrine systems, including the male and female reproductive axis (259, 260). In the past several decades, there was a populational secular trend for earlier age at pubertal development in girls and boys, as well as increasing rates of incidence and prevalence of CPP in both sexes (2, 21). These trends have been related to multiple possible factors, including environmental factors such as the exposure to EDCs (2, 21, 261). The categories of EDCs that have been associated with CPP include the following: bisphenols, phthalates, pesticides, heavy metals, essential oils, pharmaceuticals, parabens, flame retardants, and phytoestrogens (21, 259–261).

Most studies in humans linking EDC exposure to early pubertal development had retrospective or cross-sectional designs (21, 261), not identifying potential causal mechanisms. Causal mechanistic links between exposure and pubertal phenotypes have been proposed mainly by experimental animal models (21, 260). Real-world methodological difficulties of human studies include differences among the pubertal milestones evaluated (age of thelarche, pubarche, or menarche), exposure to several compounds at the same time, differences in exposure dose and duration, and differences in exposure routes. EDCs are known as hormonally active substances that function by binding to specific receptors and altering hormonal synthesis, action, and metabolism (259, 261). Many EDCs are known to present estrogenic or antiandrogenic actions. Notably, until recently, the main putative actions of EDCs were estrogenic or antiandrogenic activities, indicating a peripheral mechanism for association with pubertal outcomes (260). However, current data have suggested EDC actions on the developing brain, including the GnRH neurons. The hypothalamic GnRH network is regulated by organizational processes occurring in fetal and early postnatal life, both critical windows of development, when fetuses and neonates are vulnerable to environmental exposures, especially to EDCs (21, 260). Similarly, the timing of puberty onset is triggered by the reactivation of the HPG axis through a process regulated by genetic, epigenetic, and environmental factors, indicating that it may also be susceptible to disturbance by EDCs. Therefore, growing evidence has related these critical periods of exposure to EDCs with pubertal development disorders, based on the assumption that the neuroendocrine control of reproduction responds to environmental disruption (21, 260, 261). Nevertheless, there is no compelling current evidence that EDCs cause an up or downregulation in the GnRH and kisspeptins system (260, 261). Table 3 summarizes the main EDCs and their putative mechanisms of action associated with CPP.

A systematic review and meta-analysis evaluated the current evidence on the timing of pubertal onset in girls and boys following prenatal or postnatal exposures to xenobiotic EDCs (262). The authors demonstrated that postnatal exposure to phthalates may be associated with earlier thelarche and later pubarche. However, no consistent evidence for associations between timing of pubertal onset and exposures to any of the studied xenobiotic EDCs was found (262).

Epigenetic marks are considered players for the transmission of environmental information to physiological systems, such as the GnRH network (21, 260). Recently, epigenetic mechanisms have been hypothesized as potential targets for EDC actions in the brain, especially during development (263, 264). Animal studies have shown that exposure to distinct EDCs in critical periods of development may have disrupting effects on DNA methylation, histone posttranslational modifications, and noncoding RNA (260, 265). However, whether the effects of EDCs targeting epigenetic mechanisms modulators of pubertal timing would manifest as premature sexual development in humans is still an open field. Remarkably, regarding CPP, the body of current evidence shows a significant variability among different populations in the responsiveness to the exposure of EDCs (266). Besides differences in routes, doses, and times of exposure, the individual genetic background may also be considered as an additional factor modulating the responsiveness to EDCs exposures.

International Adoption

Various authors from different countries have analyzed a possible increase in the clinical cases of CPP among children emanating from international adoption; however, more information is clearly required. Considering the current state of knowledge, there are 4 elements that must be rigorously evaluated to confirm this working hypothesis: (1) the lack of epidemiological data in the international literature regarding CPP; (2) the information regarding the incidence of CPP among children coming from national or international adoption is only partial; (3) the available data regarding the precise chronological age in children undergoing international adoption is often deficient because of uncertainty regarding the patient's date of birth; and (4) there is enormous disagreement among clinicians as to whether the actual criteria for defining precocious puberty are appropriate.

According to The Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat that monitors population policies worldwide, approximately 260 000 international adoptions occur each year (267). International adoptions substantially increased during the past 2 decades in Western countries (268, 269), with the United States, France, Spain, and Italy having the highest number of international adoptions (1). This increased risk of CPP in internationally adopted children compared with the local population (270–274) is of great importance and the possible underlying mechanisms should be elucidated.

Nearly 60% of immigrants to Spain are from Latin America and there is a high percentage of adoptions from the same countries (8). These adopted children had more than 5 times more risk to develop CPP, all of whom were girls, with no increased risk found in those who immigrated to Spain from Latin America with their families (8). This difference in risk was not explainable by sex, genetics, or specific environmental factors; however, the BMI tended to be lower in adopted children compared with immigrants from Latin American countries (8). This may indicate increased nutritional deficiencies in adopted children (270, 274), which could modify their response to a nutritionally enriched environment. Furthermore, immigrants live with their families and most likely have less affective deprivation compared with adopted subjects (274). In this study (8), 83.8% of the children adopted abroad arrived before 6 years of age lessening the possibility of discordance in the chronological age of these children.

In a Danish cohort of children who were diagnosed with CPP between 1998 and 2017, the annual incidence of CPP and normal variants of early puberty was found to increase substantially during the past 20 years (8), with CPP increasing in immigrant girls, but with no observed in boys (1). In a

Table 3. Endocrine-disruptors chemicals and putative mechanisms of action associated with central precocious puberty

Category	Chemicals related with pubertal disorders	Main sources	Putative mechanisms involved in pubertal disorders, especially in CPP	Possible molecular targets in GnRH network in rodent models (259, 260, 261)
Bisphenols	Bisphenol A	Plasticizers, drink and food containers, building materials	- Estrogenic activity by binding to estrogen receptors - Impairment of kisspeptin neurons maturation at the arcuate nucleus	- At hypothalamic nuclei: estrogen receptor-∞, estrogen receptor-ß, kisspeptin, GABA, glutamate, RFamide-related peptide 3 - Effects on DNA methylation (*Dnmt1, Dnmt3a, Dnmt3b, Esr1*) - Effects on histone posttranslational modifications
Phthalates	Phthalates compounds	Plasticizers, consumer products (especially cosmetics), food packing, toys	- Estrogenic activity by binding to estrogen receptors - Antiandrogenic activity - Increased kisspeptin activity	At hypothalamic nuclei: estrogen receptor-∞, estrogen receptor-ß, kisspeptin
Pesticides	Persistent (DDT and DDE) and nonpersistent	Agricultural applications	- Estrogenic activity by binding to estrogen receptors - Antiandrogenic activity by altering sex steroid synthesis enzymes	- At hypothalamic nuclei: estrogen receptor-∞, estrogen receptor-ß, glutamate - Effects on DNA methylation (*Esr1*)
Heavy metal	Mercury	Diet-delivered, especially in fish and seafoods	Accumulation in the hypothalamus, the pituitary, and the gonads	
Essential oil	Lavender oil components	Lavender-fragranced products	Premature breast development: estrogenic and antiandrogenic properties of components of lavender essential oil	
Pharmaceutical	Paracetamol	Analgesics and antipyretics	- Inhibition of prostaglandins signalling possibly disrupting physiological roles of prostaglandins in the development of the brain and the gonads - Increase of estrogen production - Inhibition of androgen production	
Parabens	Parabens	Consumer products (cosmetics and personal care)	Estrogenic activity	At hypothalamic nuclei: estrogen receptor-∞
Flame retardants	Polybrominated diphenyl ethers (PBDE)	Plastics, textiles, furniture	Estrogenic and androgenic properties	
Polychlorinated biphenyl (PCB)	Mixtures	Oils, additives	Action on estrogen receptors and neurotransmitter receptors	- At hypothalamic nuclei: estrogen receptor-∞, estrogen receptor-ß, kisspeptin - Effects on miRNAs
Phytoestrogens	Genistein	Soybean, oats	Estrogenic properties	At hypothalamic nuclei: estrogen receptor-∞, estrogen receptor-ß, kisspeptin
Dioxins	Dioxin	Industrialized products	Antiandrogenic activity	

French cohort, a much higher incidence of CPP was found in girls compared with boys in sporadic and familial forms, as well as in adopted children (275). In a Korean study based on a national registry, the incidence and prevalence rates of CPP were very high (7); however, adoptees from Korea were not found to have an increased risk of precocious puberty compared with that of their country of adoption. Thus, the effect of origin might be explained, at least in part, by genetic factors linked to ethnicity or environmental factors in different countries.

It is suggested that the older the child is at adoption, the higher the risk of early puberty, with very young children at adoption having little or no increased risk of early puberty (274). Although the mechanisms underlying the relationship between international adoption and CPP remain unknown, ethnical, emotional, and environmental factors may be involved. Follow-up programs for these children starting at the time of their arrival to host countries would be of great utility. In addition, adequate nutrition should be established, avoiding excess weight gain (9) because exogenous obesity is common in occidental countries and an increased BMI is associated with early puberty (276). Indeed, girls with a BMI greater than or equal to the 85th percentile have the highest risk of both early pubertal onset and earlier age of menarche among Black, Hispanic, and Caucasian girls (277). It is possible that these adopted children have experienced various degrees of malnutrition, emotional abandonment, and illnesses, including important infectious processes, with little or no clinical information in many cases (22) that could influence the possibility of experiencing early pubertal onset or precocious puberty.

Early Exposure to Sex Steroids

Peripheral or gonadotropin-independent precocious puberty is caused by excessive sex hormones produced autonomously in the gonads or adrenal glands, β-human chorionic gonadotropin-secreting tumors, or exposure to exogenous sex hormones. It is well known that long-term exposure to sex steroids because of a GnRH-independent process can lead to CPP. This transition from peripheral to central precocious puberty usually occurs after initiation of treatment for the underlying peripheral disease, particularly in patients with significantly advanced bone age (>10 years). Once the negative feedback effect of pubertal sex steroid concentrations is removed, the hypothalamic-pituitary-gonadal (HPG) axis can subsequently be activated. In this situation, GnRH analogs are frequently necessary as adjuvant therapy in progressive CPP in combination with therapies for peripheral precocious puberty.

The development of CPP after prolonged androgen exposure has been documented in several disorders, including congenital adrenal hyperplasia, Leydig cell tumors, familial male-limited precocious puberty, and androgen-secreting adrenocortical tumors (278, 279). A retrospective study that included 63 patients with virilizing adrenocortical tumors demonstrated that 37 of them had puberty and 10 patients manifested CPP or early fast puberty (278). Tall stature and older age at diagnosis of the adrenal tumors were associated with higher risk of CPP. This study reinforces the importance of close follow-up after surgery to identify and treat consequences of early exposure to androgen excess.

Notably, CPP can be the first clinical manifestation of peripheral precocious puberty that may have a late diagnosis. This presentation was demonstrated in nonclassical congenital adrenal hyperplasia, familial male-limited precocious puberty, and McCune Albright syndrome patients (280). Nonclassical congenital adrenal hyperplasia was diagnosed in approximately 5% of girls who initially presented CPP (280).

The mechanisms that trigger secondary HPG axis reactivation are not well understood to date and the temporal correlation between skeletal maturation and HPG axis maturation may be a clue. A positive feedback mechanism from sex steroids (estrogens and androgens as well as their precursors or metabolites) on kisspeptins neurons or other key central stimulatory factors that expressed estrogen and androgen receptors could induce hypothalamic GnRH secretion leading subsequently to CPP. However, this hypothesis still needs to be demonstrated in experimental studies.

Perspectives and Conclusions

The etiology of CPP is multiple and heterogeneous, including diverse congenital and acquired causes that can be associated with different lesions or functional alterations of CNS. The importance of genetic and/or epigenetic factors in the underlying mechanisms of CPP, such as brain anomalies or tumors (hamartomas, gliomas), syndromic forms (Temple syndrome, PWS, Rett syndrome) and imprinting disorders leading to familial CPP (*MKRN3* and *DLK1* loss-of-function mutations) has grown significantly in the last decade. Indeed, the previously known high prevalence of idiopathic CPP in girls (90%) and boys (50%) should be reduced substantially when an updated genetic strategy of investigation in familial and sporadic cases with CPP is established.

The assessment of novel serum proteins (MKRN3, DLK1, KISS1, neurokinin B) through immunoassays could represent an innovative approach for CPP etiology investigation. The discoveries involving the role of these neuropeptides in determining premature reactivation of HPG axis, or even as biochemical screening for further mutational gene analysis in selected CPP patients, are open fields to be explored (11, 63, 192, 281–283).

Exposure to EDC either prenatally or postnatally during childhood has been proposed as possible mechanisms for the occurrence of CPP (21, 260). However, the effects of EDC are difficult to determine because they are compound specific, sexually dimorphic, and time exposure dependent (260).

Despite the successful outcome after CPP treatment with long-acting GnRH analogs, the recent genetic discoveries involving the etiology of CPP may provide the bases for potential new target treatments in specific and selected subgroups.

Girls with early or precocious abnormal puberty have longer chronic estrogen exposure than girls with normal pubertal development. They have potentially an increased subsequent risk of obesity, type 2 diabetes mellitus, cardiovascular disease, breast cancer, and other malignancies (284, 285). In addition, the early sexual development is also associated with more frequent risk-taking behaviors (286).

Because CPP has been linked to changes in body weight and obesity, exposure to EDC, and stressful life events, preventive measures to avoid these conditions, such as healthy living habits, are highly recommended.

The identification of the major causative factors (genetic or environmental players) involved in the regulation of the hypothalamic neural network that modulate GnRH secretion will positively influence prevention, early and precise diagnosis, and better outcomes of children who experienced precocious sexual development.

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The authors have nothing to disclose.

Data Availability

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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4.5 – CAPÍTULO 4 – "FAMILIAL CENTRAL PRECOCIOUS PUBERTY DUE TO *DLK1* DEFICIENCY: NOVEL GENETIC FINDINGS AND RELEVANCE OF SERUM DLK1 LEVELS"

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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

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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)
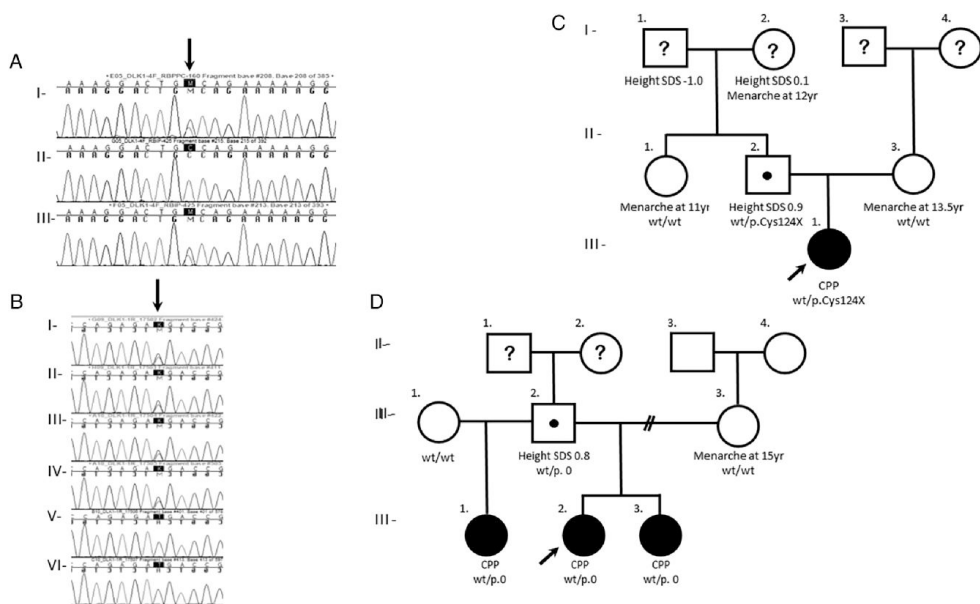

Figure 1. Pedigrees of the 2 families and sequencing data of the *DLK1* gene showing rare allelic variants. A. Electropherogram of index patient 1 and her relatives. I) Index case harboring the *DLK1* pathogenic allelic variant c.372C>A; p. Cys124X—(rs749564412). II) Normal electropherogram of her mother. 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
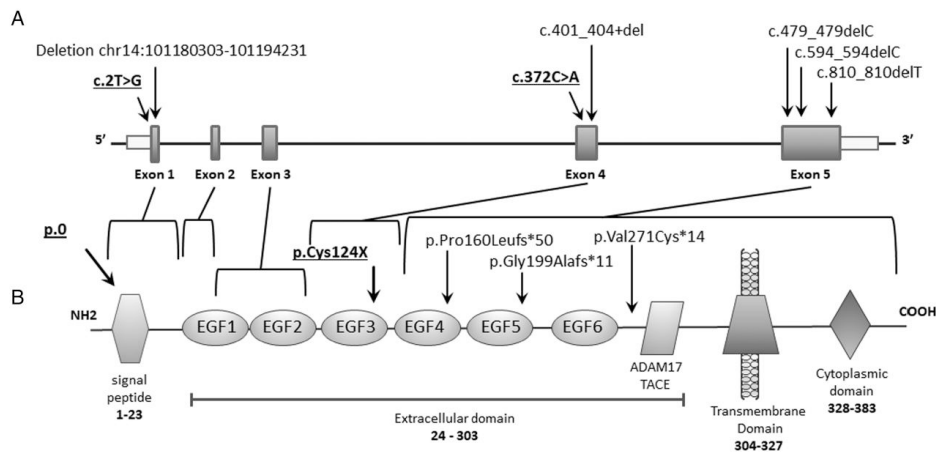

Figure 2. Schematic representation of the human *DLK1* gene and human DLK1 protein. A. Human *DLK1* gene (transcript ENST00000341267.9): The boxes indicate the coding sequences of the 5 exons of the gene in humans. Open boxes indicate the 5′- and 3′-untranslated regions of the gene, respectively. Previously 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

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.

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.

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
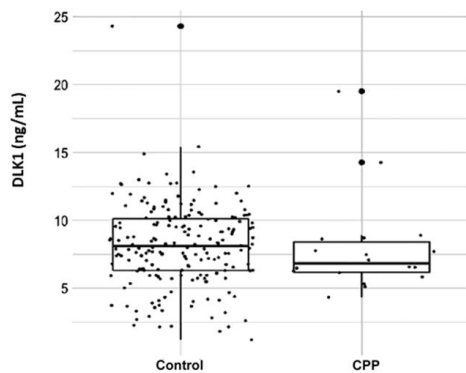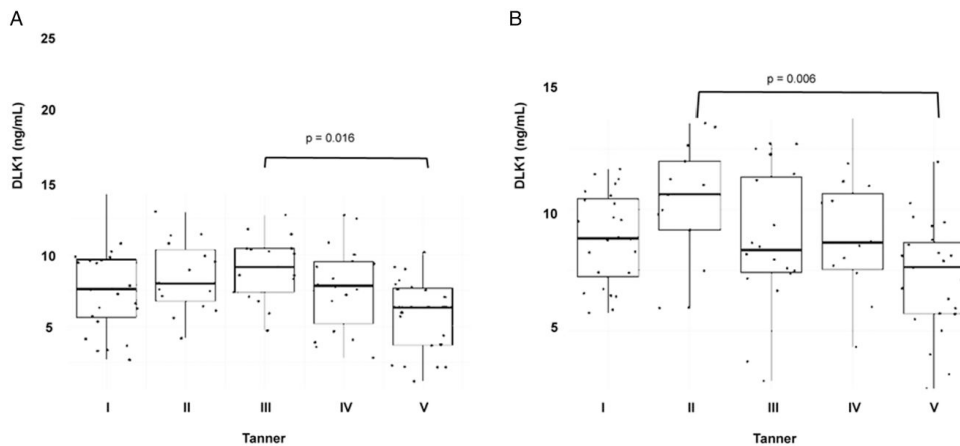

Figure 4. DLK1 levels in girls and boys according to Tanner stages. DLK1 levels according to puberty Tanner stages in (A) girls and (B) boys.
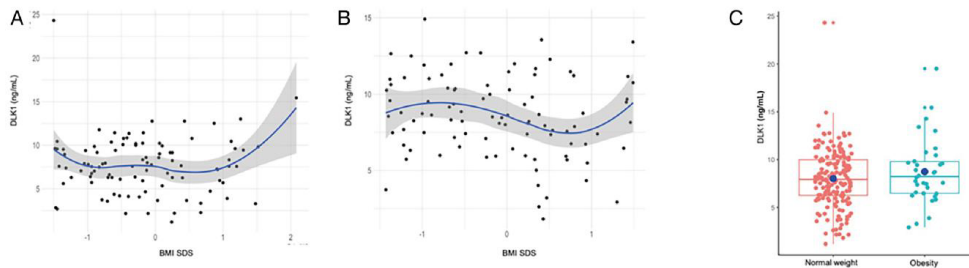
Figure 5. DLK1 according to BMI SDS and age. A. DLK1 levels vs BMI SDS in girls. B. DLK1 levels vs BMI SDS in boys. C. DLK1 levels in obese/overweight vs normal weight subjects. BMI, body mass index.
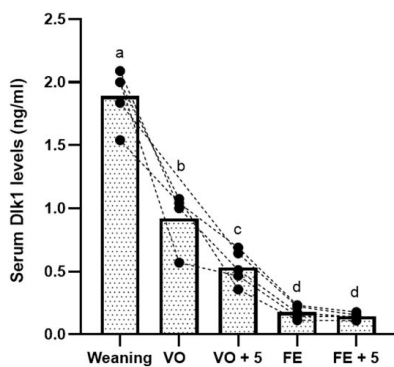

Figure 6. Circulating serum Dlk1 levels in peripubertal female mice. Serum Dlk1 levels of 5 female mice followed longitudinally across pubertal maturation. The mice were all weaned at age 21 days. Vaginal opening (VO) occurred at age 25.6 ± 1.9 days (mean ± SEM), and first estrous (FE) at age 37.6 ± 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.

Funding

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4.6 – CAPÍTULO 5 - UNRAVELING THE METABOLIC CONSEQUENCES OF *DLK1* DEFICIENCY IN WOMEN WHO HAD CENTRAL PRECOCIOUS PUBERTY DURING INFANCY

* Artigo em processo de submissão

Unraveling the metabolic consequences of DLK1 deficiency in women who had central precocious puberty during infancy

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Abstract

Background: Loss-of-function mutations of Delta-like Noncanonical Notch Ligand 1 gene (*DLK1*) have been implicated in central precocious puberty (CPP) and metabolic syndrome.

Objective: To explore the metabolic impact of *DLK1* deficiency in women who had the diagnosis of CPP caused by defects in this gene. We investigated their adipose tissue distribution, the consequences over adipogenesis and mitochondrial function impact.

Patients and Methods: Seven women (mean age of 43.2 ± 21.6 yrs and mean BMI of 29.9 ± 6.8 kg/m^2) who carried loss-of-function mutations of DLK1 were extensively evaluated. Assessments included body composition via bioelectrical impedance analysis and abdominal CT scans. Peripheral blood analyses for mitochondrial function and oxidative stress were performed and compared with six healthy subjects. Additionally, 3T3-L1 cells were submitted to an adipocyte differentiation protocol with serum from either group and subsequently, the amount of fat droplets were compared.

Results: The DLK1 deficient women exhibited increased visceral adipose tissue (VAT) and higher VAT/subcutaneous adipose tissue ratio. Mitochondrial mass and oxidative stress were markedly elevated in DLK1 deficient women, and their serum led to increased adipogenesis from precursor cells. Their leukocytes showed decreased oxygen consumption rate and increased extracellular acidification rate. Two DLK1 deficient women submitted to a weight loss regimen successfully lost a significant amount of fat mass.

Conclusions: This study provides compelling evidence of the distinct metabolic profile in women with *DLK1* loss-of-function mutations. Absence of DLK1 is permissive to adipose tissue expansion and a worse metabolic phenotype.

Introduction:

Recent discoveries have shed light on loss-of-function mutations in Delta-like Noncanonical Notch Ligand 1 (DLK1) among patients with previously unexplained central precocious puberty (CPP) (1). In 2017, Dauber A. *et al*. (2) identified a complex deletion-duplication mutation resulting in a truncated DLK1 protein in a Brazilian family with CPP. This was further expanded by Larissa G *et al* (2)*,* who described three distinct frameshift mutations in 10 women who had diagnosis of familial CPP from Brazil and UK (1,2). Notably, this group exhibited a higher prevalence of metabolic syndrome compared to either 20 idiopathic CPP subjects or other CPP cohorts previously characterized (2). Further studies have also linked DLK1 mutations with CPP, though metabolic syndrome features were not universally present (3–6). For instance, two French and one Spanish girls, in separate studies, displayed no significant metabolic symptoms (3,4). However, one Chinese boy and one Italian carrier father had hyperuricemia and dyslipidemia, (4,6). Altogether, an analysis of all 17 reported cases to date, focusing on the 12 individuals under 25 years, reveals that 50% exhibited one or more metabolic syndrome components – a rate unexpectedly high for this age group and for the general population (7).

DLK1 is an inhibitor of Delta-NOTCH pathway, which is highly conserved in mammals (8,9). It features an extracellular domain comprising six epidermal growth factor (EGF)-like repeats, a TNF-α-converting enzyme (TACE/ADAM17) cleavage site, a transmembrane domain, and a short intracellular domain (8,10). The extracellular domain of DLK1, measurable in serum, was found to be undetectable in all patients in a study by Gomes L *et a*l (2). DLK1's interaction with NOTCH receptors leads to inhibited signaling. The mechanism by which the loss of DLK1 leads to premature pubertal development is not fully understood. However, DLK1's role in inhibiting adipocyte differentiation and its association with metabolic syndrome characteristics is well established (11,12). It notably maintains pre-adipocytes in their undifferentiated state. Furthermore, DLK1 serum levels have been positively correlated with body mass index (BMI), adipose tissue mass, and insulin resistance in adults (13,14). Contrarily, in younger individuals, the correlation is less consistent, but there seems to be a progressive decrease in DLK1 levels with puberty progression (5).

89   In metabolic syndrome, one of the remarkable features is white adipose
90   tissue disfunction and inflammation. The white adipose tissue in patients with
91   metabolic syndrome typically contains a higher number of macrophages with an
92   inflammatory phenotype compared to lean individuals (ref). This pattern is even
93   more pronounced in the visceral adipose tissue, which is known to have more
94   metabolic and inflammatory activity (15). In addition, metabolic syndrome is also
95   associated with mitochondrial dysfunction and chronic oxidative stress (16,17).
96   The specific effects of DLK1 deficiency on adipose tissue morphology,
97   function, and composition remain unclear. Studies on murine models with *Dlk1*
98   knockout have indicated increased susceptibility to weight gain, characterized by
99   augmented white adipose tissue, steatosis, and insulin resistance (11,18).
100   Furthermore, inhibition of NOTCH signaling has been linked to "browning" of
101   adipose tissue (19). Brown adipose tissue is associated with greater energy
102   expenditure and better metabolic phenotype (19). Additionally, NOTCH activation
103   is known to impair mitochondrial respiration, increase superoxide production and
104   AMPK activity (20). Notably, animal studies suggest that NOTCH signaling can
105   induce the activation of proinflammatory M1 macrophages and heighten
106   mitochondrial oxidative stress (21). The current study is designed to explore the
107   effects of DLK1 deficiency on white adipose tissue characteristics and
108   inflammation in humans.
109
110   **Methods**
111
112   **Patients**
113
114   All study participants provided written informed consent, and the research
115   was approved by the local ethics committee. This study analyzed seven subjects
116   with confirmed *DLK1* mutations who were followed on a single center (Hospital
117   das Clínicas, Brazil) (2). The mutations found in those patients are described in
118   **Supplementary Table 1**. Six of these subjects underwent imaging studies to
119   assess the distribution of their white adipose tissue. Additionally, five of these
120   subjects provided peripheral blood samples for further metabolic analysis, with
121   data compared against a control group of six healthy females. Furthermore, two
122   subjects (Patients 1 and 2) participated in a two-year weight loss program

123   involving sibutramine treatment and biannual nutritional counseling. We

124   conducted sequential body composition analyses to monitor changes throughout

125   their weight loss.

126

127   **DNA Sequencing**

128   For genetic analysis, genomic DNA was extracted from peripheral blood

129   lymphocytes of all cases, using established protocols. The *DLK1* gene (5 exons—

130   GenBank accession number NM_003836) was amplified by polymerase chain

131   reaction (PCR). PCR products were then purified and sequenced using the

132   Sanger method. DNA sequences obtained were compared to the human

133   GenBank *DLK1* sequence using Sequencher sequence alignment software

134   (Gene Codes Corporation, Ann Arbor, MI). PCR primers and conditions are

135   available upon request.

136

137   *Imaging studies*

138   For body composition assessment, we utilized Bioelectrical Impedance

139   Analysis (BIA) with InBody 720 (Biospace Co., Ltd, Seoul, Korea), yielding

140   measurements of fat-free mass, body fat mass, percentage of body fat, and

141   skeletal muscle mass. Additionally, all six DLK1 subjects underwent abdominal

142   computerized tomography (CT) to quantify their visceral fat area. They also

143   underwent coronary CT scans to determine their coronary calcium score (CCS).

144   These CT scans were consistently analyzed by the same radiologist, with

145   subjects positioned supine and images captured in 10 mm increments at the L4-

146   L5 region (Helical Picker PQ 5000, Cleveland, OH, USA). Images analysis was

147   performed using Osirix Imagin Software (Pixmeo, Switerland, v3.9). For adipose

148   tissue quantification and characterization, we used a -150 to -50 Hounsfield Units

149   (HU) range for marking both visceral abdominal tissue (VAT) and subcutaneous

150   adipose tissue (SAT). Coronary CT data were processed in 2.5 mm increments,

151   synchronized with heart rate, using a 0.4-second rotation and 6 acquisitions per

152   second. The Agatston score was applied to determine CCS, with scores over 0

153   deemed positive (22). CCS were categorized as follows: 0, 0 to 100, and over

154   100 Agatston units (AU). Data was compared to established guideline cutoff

155   values and large cohorts of healthy subjects (15,23–26).

156

Peripheral blood metabolic analysis

158   Peripheral blood samples were collected from five DLK1 patients and six

159 control subjects using EDTA tubes. These samples were centrifuged at 700x G

160 for 30 minutes to separate plasma. From the leukocyte fraction, we isolated

161 peripheral mononuclear cells (PBMCs) and granulocytes (neutrophils) for further

162 analysis.

163

164 <u>Flow cytometry</u>

165   The cell fractions were washed in PBS and stained with 200nM

166 MitoTrackerGreen, 50nM MitoTrackerDeepRed and 5 mM MitoSOX (Invitrogen,

167 Carlsbad, CA, USA). Analysis was conducted using a flow citometer

168 (FACSCanto) and BDFACSDiva software (BD Biosciences, San Diego, CA,

169 USA), with data processing in FlowJo software (FlowJoLLC, Ashland, OR, USA).

170

171 <u>Macrophage differentiation</u>

172   Macrophage polarization was induced by incubation with GM-CSF (25

173 ng/mL) and IL-4 (10ng/mL) for 9 days and subsequent marking for flow cytometry

174 Details are provided in **Supplementary Table 2**.

175

176 <u>Pre-adipocytes differentiation</u>

177   3T3-L1 cells (ABCAM, BOS, USA) were submitted to a pre-adipocyte

178 differentiation protocol outlined in **Supplementary Table 3**. Five cultures treated

179 with serum from affected subjects and six with serum from control subjects were

180 stained with Oil Red O (ab150678, ABCAM, USA) for optical microscopy analysis.

181

182 <u>Extracellular flux analysis (Seahorse)</u>

183   Live human leukocytes, separated from peripheral blood samples of both

184 DLK1 and control groups, were analyzed for oxygen consumption rate (OCR) and

185 extracellular acidification rate (ECAR) using a Seahorse XF 96 bioanalyzer

186 (Agilent,USA). The experiments used 1mg/ml oligomycin (SigmaAldrich), 5mM

187 FCCP (Sigma-Aldrich), 1mg/ml antimycinA (Sigma-Aldrich), 25mM glucose

188 (Sigma-Aldrich) and 20mM 2-DG (Sigma-Aldrich). These substances were

189 injected to obtain maximal respiratory and control values. Assay parameters were

190   as follows: 3min mix, no wait, 3min measurement, repeated 3 times at basal and
191   after each addition.

192

193   **Statistical Analysis**

194   For statistical analysis, we utilized descriptive statistics to calculate
195   frequencies and percentages for all numerical and categorical variables. Data
196   were primarily presented as mean ± standard deviation (SD) unless otherwise
197   stated. We used Student's t-test or the Wilcoxon signed-rank test for continuous
198   variables, and the Chi-square test or Fisher's exact test for categorical variables,
199   as appropriate. Statistical analyses were conducted using R Studio (version
200   1.2.1335) or Python (v 3.12.0). A p-value less than 0.05 was considered
201   statistically significant.

202

203   **Results**

204

205   *Imaging studies for DLK1 CPP*

206   Among the six female patients with *DLK1* mutations who performed
207   abdominal CT three (50%) had increased VAT and two (33%) had increased
208   SAT/VAT ratio. None of the patients exhibited an increased CC (Table 1). **Figure
209   1A** illustrates a subject with increased VAT and SAT/VAT ratio, while **Figure 1B**
210   shows a subject with normal VAT and SAT/VAT, though increased subcutaneous
211   adipose tissue is noticeable.

212

213   *Weight loss during obesity treatment*

214   Among the two subject who engaged in the weight loss therapy, the first
215   lost 17.4 kgs (18.6% of initial weight) and the second lost 12.9 kgs (17.7% of
216   initial weight). The weight loss was predominantly from fat mass **(Figure 2).**

217

218   ***Peripheric blood metabolic analysis***
219   The clinical data for the eleven patients (5 DLK1 and 6 controls) who
220   underwent peripheral blood metabolic function evaluation is summarized in **Table
221   2.** It should be pointed that the marginally younger age and lower BMI observed

7

222 in the control group were not statistically significant, and these figures were
223 skewed by an outlier, specifically subject DLK1-3. Upon exclusion of this outlier,
224 the mean age in the DLK1 group adjusts to 35.7 years as opposed to 34.8 years
225 in the control group, and the mean BMI shifts to 27 kg/m^2 versus 25.2 kg/m^2 in
226 controls. Importantly, the inclusion of this outlier did not affect the validity of our
227 results, as the data from DLK1-3 were not utilized in the statistical analyses.
228

229 *Metabolic evaluation of mitochondrial mass and oxidative stress*

230 Using flow cytometry, as depicted in **Figure 3A**, we observed a significant
231 increase in mitochondrial mass (MitoTracker Green staining) and mitochondrial
232 membrane potential (MitoTracker DeepRed) in DLK1 subjects. Additionally,
233 lymphocytes and monocytes from these subjects exhibited heightened SOX
234 production, as shown in **Figure 3B.**

235

236 *Macrophage differentiation*

237 Analysis of M1 inflammatory macrophage differentiation revealed an
238 elevated presence of the CD44 marker in DLK1 subjects compared to controls.
239 However, for M2 markers, the differences between the two groups were not
240 statistically significant **(Figure 4).**

241

242 *Pre-adipocytes differentiation*

243 Treatment of cells with serum from DLK1 subjects resulted in increased
244 formation of mature adipocytes, characterized by larger oil red-stained lipid
245 droplets. These observations are illustrated in **Figure 5**, captured with a 50μm
246 resolution.

247

248 *Extracellular flux analysis (Seahorse)*
249 Peripheral PBMCs from DLK1 subjects demonstrated a reduced oxygen
250 consumption rate (OCR) and a similar extracellular acidification rate (ECAR) to

251   controls. As for the PMNs (neutrophils), those obtained from DLK1 subjects had

252   a reduced OCR and a heightened ECAR **(Figure 6).**

253

254

255

256

257

Discussion

DLK1 is a classical inhibitor of adipocyte differentiation and has recently been implied in pubertal timing. The current study adds crucial insights to the understanding of DLK1 loss-of-function mutations as a rare but significant cause of central precocious puberty (CPP) and reinforces its link with metabolic syndrome (MS). Through the analysis of nine adult women with *DLK1* mutations from the limited global pool of reported instances – 17 reported cases to date - we have identified pronounced cellular metabolic abnormalities and an inclination towards inflammatory macrophage polarization in individuals harboring *DLK1* mutations. These findings underscore the systemic implications of such genetic mutations.

A visceral adipose tissue (VAT) area greater than 100 cm^2 and VAT/SAT ratio greater than 0.4 have been correlated to higher cardiovascular risk (23,27). We identified 50% and 33% of young patients with *DLK1* mutations with increased VAT and VAT/SAT ratio, respectively. *DLK1* patients also had an elevated average SAT area, with a mean value of 355.8 ± 119.1 cm^2 for individuals averaging 40.6 ± 19.7 years. Although most studies demonstrate a strong correlation of VAT with cardiovascular risk, increased subcutaneous adipose tissue (SAT) has also been correlated, to a lesser extent, to these outcomes (28). SAT area in *DLK1* patients contrasts with larger studies conducted in the general population, such as a Spanish study of an older age range (mean age 57.7 ± 10.2 years) that described a significantly lower average SAT of 213.6 ± 120.2 cm^2 (26). Likewise, an international study encompassing 4144 subjects, with an average female age of 57-58 years, indicated an average SAT around 300 cm^2 (29). These findings suggest a distinct metabolic profile in patients with *DLK1* deficiency, characterized by increased VAT area, elevated VAT/SAT ratio, and higher SAT levels in relation to the general population. Nevertheless, the normal coronary calcium scores observed may be indicative of the cohort's younger average age relative to the temporal lag typically preceding the onset of cardiovascular events.

Furthermore, the impact of *DLK1* mutations on adipogenesis was corroborated by the enhanced differentiation of adipocytes *in vitro*, when cell cultures were treated with serum from affected individuals. This is to be expected,

292 given that serum from affected individuals had undetectable levels of DLK1, a
293 protein known to act as a critical regulator of adipogenesis by inhibiting the
294 differentiation of pre-adipocytes (12). This propensity for adipose tissue
295 expansion, presumably exacerbated under conditions of excess caloric intake,
296 aligns with previous observations from animal studies (11). Notably, two women
297 with DLK1 deficiency lost weight (> 15% of initial weight) under hypocaloric diet
298 and obesity drug treatment interventions, suggesting that DLK1's role in
299 adipogenesis is permissive rather than determinative and that fat tissue
300 expansion is reversible in these subjects.

301 Beyond adiposity, the *DLK1* mutation group also demonstrated evidence
302 of impaired mitochondrial respiratory function and a shift towards M1
303 macrophage polarization compared to healthy controls.

304 Peripheral leucocytes in the DLK1 group showed an increased
305 mitochondrial mass with an increased production of free radicals such as SOX.
306 As a matter of fact, SOX production in peripheral leukocytes from this group
307 approached levels seen in positive controls treated with hydrogen peroxide,
308 underscoring the profound oxidative stress associated with DLK1 loss.

309 These findings dovetail with two interlinked evidences: firstly, the well-
310 established connection between metabolic syndrome and systemic inflammation,
311 which in turn is related to adipose tissue macrophage dysfunction; secondly, the
312 role of NOTCH signaling in oxidative stress, which is exacerbated by the absence
313 of its inhibitor, DLK1 (30,31). NOTCH signaling knowingly increases oxidative
314 stress (21). Furthermore, this pattern is consistent with our findings of reduced
315 oxygen consumption rate (OCR) and increased extracellular acidification rate
316 (ECAR) in live cells.

317 Although the study shows a single elevated marker for M1 macrophage
318 differentiation, this finding should be viewed with caution due to the inconclusive
319 nature of the other markers. Nonetheless, the constellation of metabolic
320 consequences presented herein would be consonant with an increased M1
321 polarization.

322 In conclusion, our study provides compelling evidence of the distinct
323 metabolic profile in patients with *DLK1* loss-of-function mutations. The higher
324 prevalence of cardiovascular risk factors, such as elevated VAT and SAT areas
325 and their ratios in these patients, underscores the potential for *DLK1* mutations

326 to significantly impact metabolic health. Furthermore, the unexpected high levels
327 of oxidative stress in peripheral leukocytes of these patients and the impaired
328 mitochondrial function highlight the broader systemic effects of these mutations.
329   Future research should focus on elucidating the precise mechanisms by
330 which DLK1 influences adipose tissue distribution and metabolic pathways.
331 Additionally, exploring potential therapeutic interventions to mitigate the
332 metabolic consequences of *DLK1* loss-of-function could be highly beneficial. This
333 study opens new avenues for understanding the complex interplay between
334 genetic factors and metabolic health, paving the way for more targeted and
335 effective treatment strategies in CPP and metabolic syndrome.
336
337

Declaration of interests
339 The authors declare no competing interests.
340

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448   and insulin resistance in obesity. American Journal of Physiology-Endocrinology and
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452

453

454 **Tables**

455 **Table 1 –** Abdominal CT adipose tissue quantification and coronary CT results in

456 subjects with *DLK1* mutations.

| Subject | Abdominal CT | | | | Coronary CT |
	Age (yrs)	VAT (cm^2)	SAT (cm^2)	VAT/SAT ratio	Agatston
1A	37	163*	241	0.68**	0
3B	59	145*	493	0.29	0
4C	24	72.6	514	0.14	0
4B	26	28.8	302	0.10	0
4A	27	67	331	0.20	0
4E	71	178*	254	0.70**	0
Mean (SD)	40.7 (19.7)	109.1 (60.8)	355.8 (119.1)	0.35 (0.27)	0

457

458 * VAT above 100 cm^2** VAT/SAT ratio above 0.4

459

460 **Table 2 –** Clinical data for all ten patients who underwent metabolic evaluation.

Group	Subject ID	Age (Years)	Weight (kgs)	Height (m)	BMI (kg/m^2)
DLK1	DLK1-1 (4B)	28	60	1.59	23.7
	DLK1-2 (4C)	26	76	1.59	30.2
	DLK1-3 (4E)	73	79	1.38	41.3
	DLK1-4 (3B)	59	68	1.56	27.7
	DLK1-5 (3A)	30	65	1.57	26.5
	mean	43.2 (21.6) †	---	---	29.9 (6.8) ††
CTRL	CTRL1	33	54	1.58	21.6
	CTRL2	20	96.6	1.71	33.1
	CTRL3	45	57	1.63	21.5
	CTRL4	46	67	1.64	24.9
	CTRL5	24	70	1.55	29.1
	CTRL6	41	57	1.64	21.2
	mean	34.8 (11) †	---	---	25.22 (4.9) ††

461
462 † p = 0.46. †† p = 0.24. BMI = Body mass index. Data is presented as mean (standard deviation).
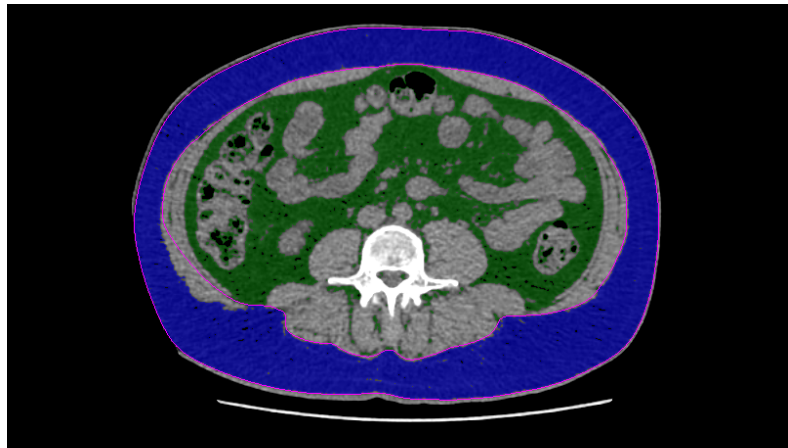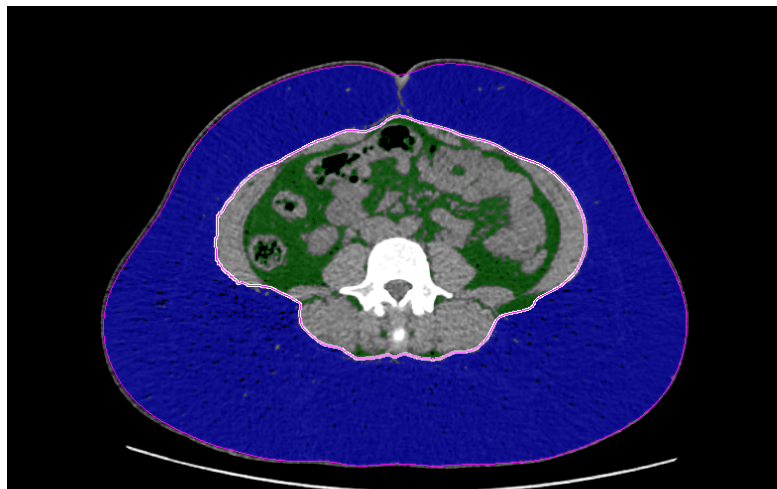463

464

465 **Figures**

466 **Figure 1 –** Abdominal CT evaluation of two subjects with DLK1 mutations

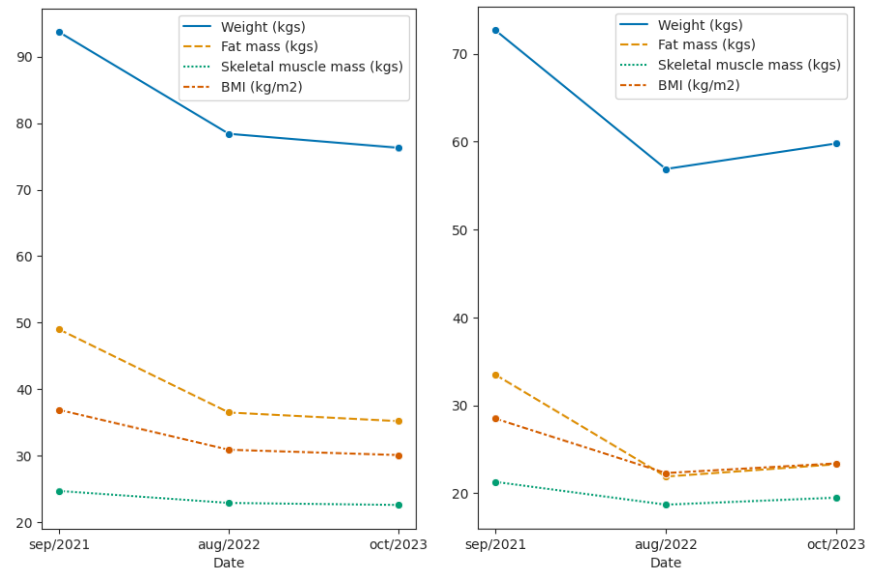467 **A)**

468

469 **B)**

470

471 Legend: Blue colored areas represent the subcutaneous adipose tissue(SAT); green
472 colored areas represent the visceral adipose tissue (VAT).

473

474   **Figure 2 –** Weight and body composition evolution of two subjects with DLK1
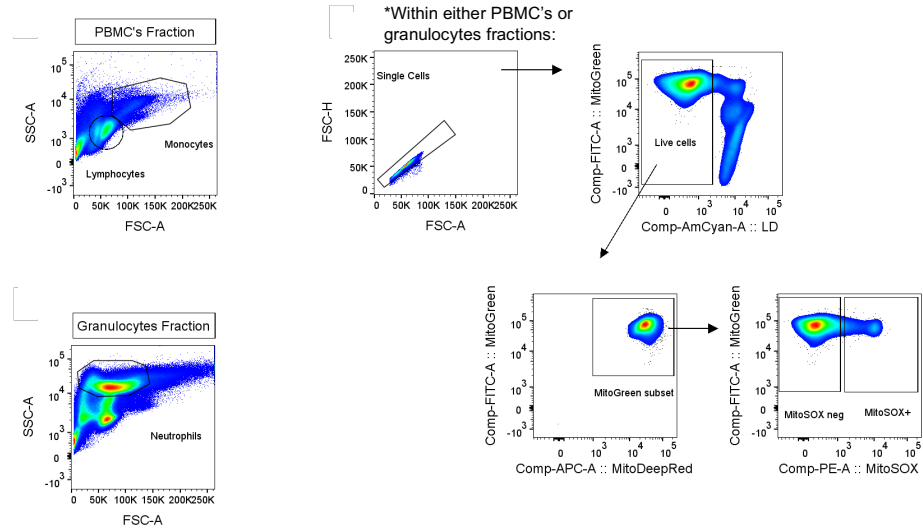475   mutations under weight loss therapy
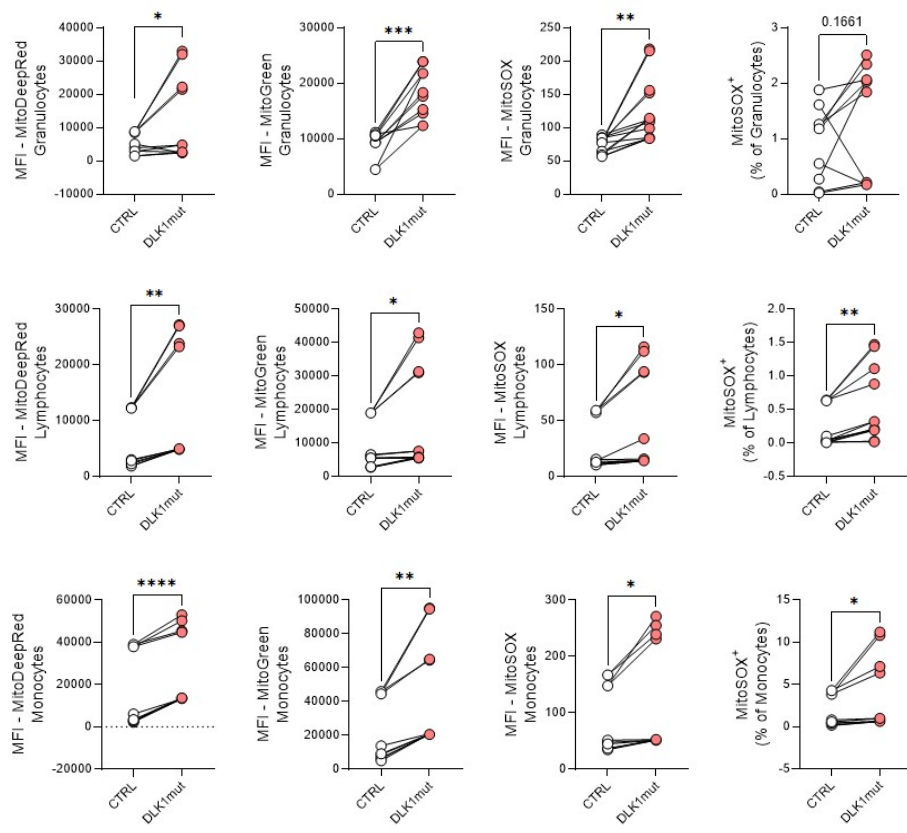

476

477

478   Legend: BMI – Body mass index

479

480   **Figure 3** – Evaluation of mitochondrial mass and oxidative stress

481   **A**

482

483   **B**

484

485

486

487 Legend: A – Flow cytometry was used sequentially to isolate PBMCs and granulocytes and to
488 perform different staining. MitoGreen stain binds to cardiolipin and correlates positively with
489 mitochondrial mass. MitoDeepRed correlates positively with mitochondrial membrane potential.
490 MitoSOX correlates with the mitochondrial production of superoxide. B- All leukocytes from
491 DLK1 mutated subjects had increased mitochondrial mass with increased membrane potential,
492 which mark mitochondrial stress. With the exception of granulocytes, all other leukocytes from
493 DLK1 subjects showed increased production of SOX. FSC-H – forward scatter height; FSC-A –
494 forward scatter area

495

496 **Figure 4 –** Macrophage markers for M1 (red) and M2 (blue) polarization

497

498

499

500
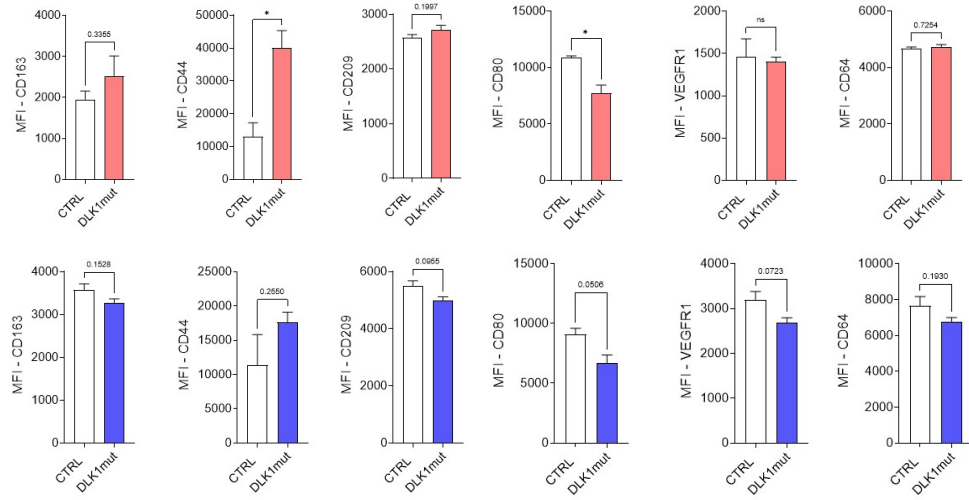
501 Legend: Colored columns depict data from the DLK1 group. The first row (red) refers to markers
502 of M1 macrophage activation. The only statistically significant marker for M1 activation found to
503 be increased in the DLK1 group was CD44. The second row (blue) refers to markers of M2
504 macrophage activation and no significant difference was found between both groups.

505

Figure 5 – 3T3-L1 cells differentiation into adipocytes when treated with DLK1 mutation subjects' serum vs controls' serum.

Controls
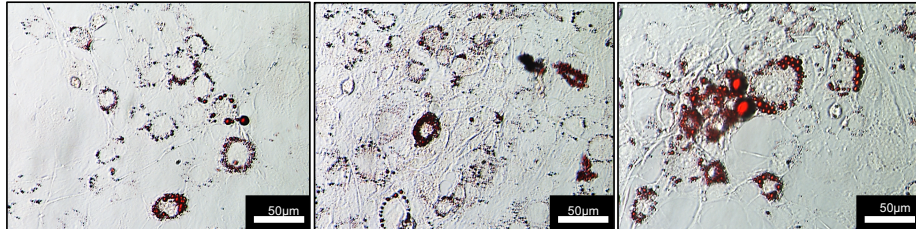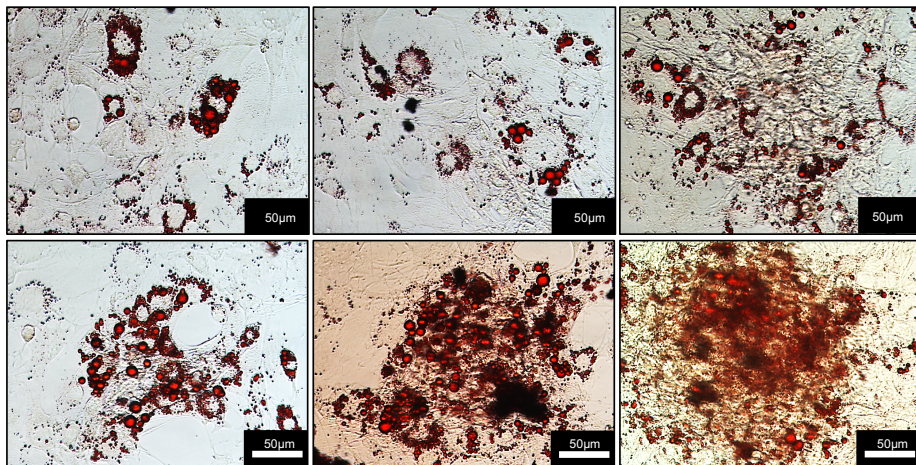
DLK1 subjects' serum

Legend: Oil red stains fat droplets. The amount of red staining in cells treated with DLK1 subjects' serum was significantly higher, denoting increased adipogenesis.

516 **Figure 6 –** Evaluation of peripheral leukocytes OCR and ECAR from subjects

517 with *DLK1* mutations and healthy controls

518

519

520 Legend: OCR - oxygen consumption rate; ECAR: extracellular acidification rate

521

522

Supplementary tables

524

Supp table 1 – Phenotype and genotype of the seven DLK1 subjects evaluated.

526

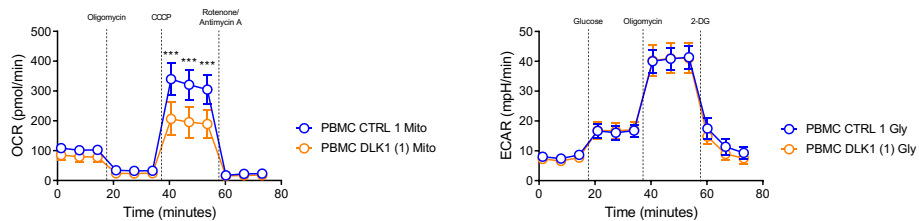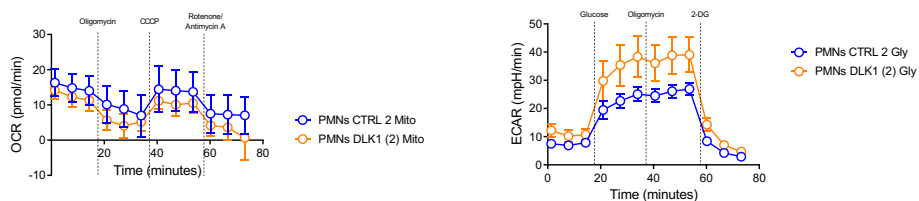| Subject	Sex (M or F)	Age (Years)	Additional Clinical Features	Mutation (cDNA) (protein)
1A	F	37	PCOS, infertility, type 2 diabetes (27yrs) and nonalcoholic fat liver disease	c.594_594delC p.Gly199Alafs*11
3A	F	30	---	c.479_479delC p.Pro160Leufs*50
3B	F	59	Glucose intolerance and dyslipidemia	
4A	F		---	Complex defect. With exon 1 deletion and 5'UTR Truncated protein
4B	F	28	---	
4C	F	26	Glucose intolerance	
4E	F	73	Type 2 Diabetes, hypertension and dyslipidemia	

527

Alternating colors indicate distinct families

529

Supp table 2 – Markers used in macrophage flow citometry

531

Marker	Target
FITC	CD64
PerCP	CD209
PE	CD16
PECy7	HLA-DR
APC	CD86
BV510	LD

532

Supp table 3 – 3T3-L1 adipocyte differentiation protocol

534

Step	Procedures
Mean preparation	-1.: 90% confluent cells DMEM + 10%FBS + 5ug/mL insulin
Cell implantation	Plaques with $3x10^{-3}$ cells/cm^2 density
Day 0	DMI cocktail (DEX 1uM; IBMX 0.5mM; regular human insulin 5ug/mL);
Day 2 to day 8	DMEM + 10%FBS + 5ug/mL insulin Changed Every 2 days
Day 9	Supernatant collection and Oil Red staining

535

5. DISCUSSÃO

Uma das grandes preocupações da PPC é o risco adverso quanto às características metabólicas. Tal preocupação decorre, em grande parte, da conclusão de estudos de GWAS que correlacionam a idade mais precoce de menarca com uma série de riscos metabólicos (16,83). Além disso, diversos estudos demonstram IMC aumentado em pacientes com PPC antes, durante e após o tratamento (84–86).

Previamente, a maior coorte de PPC avaliada quanto aos parâmetros metabólicos durante o tratamento com bloqueio puberal havia avaliado 142 mulheres adultas (entre 27 e 50 anos) com PPC, das quais 100 haviam sido tratadas com análogos de GnRH, e as comparou com 413 mulheres sem PPC (controle). Este estudo não descreveu maior prevalência de obesidade ao longo do tratamento, bem como na vida adulta em pacientes com PPC (87).

Uma análise de dados de 29 pacientes com PPC causada por mutação no MKRN3, das quais 11 tinham dados após o término do tratamento, e acompanhadas durante o presente estudo foi publicada em 2019 (86), mostrando não haver excesso de desfechos metabólicos ao longo do tratamento. Adicionalmente, em 2021 também se publicou o resultado da avaliação de 22 pacientes com PPC idiopática acompanhadas ao longo do tratamento e, novamente, não havia aumento de desfechos metabólicos ao longo do tratamento.

No segundo artigo dos resultados dessa tese, que representa a maior coorte de *MKRN3* com avaliação genética em um único centro, demonstrou-se que a PPC causada pelo *MKRN3*, quando comparada a PPC idiopática não tem um fenótipo metabólico mais pronunciado, e o IMC médio é semelhante. Na coorte acompanhada durante esse trabalho, na apresentação inicial, antes do início do tratamento, 30% das pacientes apresentavam sobrepeso e 8,6% apresentavam obesidade. Tais números são mais elevados que a prevalência de sobrepeso e obesidade na população brasileira. Embora tal dado possa ser subnotificado, os dados da Pesquisa Nacional de Saúde e os relatórios de 2019 do Sistema de Vigilância Alimentar e Nutricional mostravam 16,33% de sobrepeso e 14,6% de obesidade entre crianças com 5 a 10 anos de idade. A prevalência de sobrepeso e obesidade, no entanto, não aumentou e

até pareceu diminuir, embora sem valor estatístico, após o tratamento, em linha com os estudos citados anteriormente – encontramos 25% de sobrepeso e 7,6% de obesidade após 1 ano do tratamento. Comparando, em seguida, os dados das 184 pacientes tratadas com agonista de GnRH versus os dados pareados por idade das 56 pacientes não tratadas, demonstramos que o tratamento não incorre em maior risco metabólico, ao menos durante o tempo de tratamento e 1 ano após este.

Sendo assim, do ponto de vista clínico, as mutações do *DLK1* estão acompanhadas de maiores taxas de obesidade e parâmetros da SM. No quarto artigo, descreve-se um padrão interessante entre os níveis de DLK1 ao longo dos diferentes estágios puberais, de forma a elevar-se até o Tanner 2 em meninos e até o Tanner 3 em meninas, com posterior decréscimo progressivo. Este dado é concordante com a dosagem de Dlk1 em camundongos em diferentes fases do desenvolvimento puberal que é apresentada no mesmo artigo.

Embora a dosagem de DLK1 sérica não pareça se correlacionar, nesta casuística com pacientes em média muito jovens, com o IMC, outros estudos menores, mas que avaliaram uma população mais velha adulta (mediana de 68 anos de idade) e maior quantidade de grandes obesos demonstraram aumento dos níveis de DLK1 de acordo com o aumento de peso e de acordo com o aumento de massa gorda (73). Um artigo italiano com uma casuística menor e idade média de 12 anos descreveu o mesmo padrão, mas sem avaliar o estadiamento puberal. Sendo assim, os dados indicam que a correlação com os níveis séricos de DLK1 e obesidade/SM não é tão nítido na adolescência, inclusive por haver uma queda fisiológica, como demonstramos, ao completar o desenvolvimento puberal, mas ser mais notado ao longo da vida adulta. O aumento de DLK1, que exerce função parácrina e endócrina através de sua porção extracelular solúvel seria uma possível medida compensatória ao ganho de gordura, em indivíduos sem mutações no *DLK1*, por ser um conhecido fator anti-adipogênico. Esta resposta biológica, hipoteticamente, poderia levar a uma inibição da expansão do tecido adiposo.

Quando se comparam os dados das pacientes com mutação do *DLK1*, conforme previamente publicado por Gomes L *et al*. (26) encontra-se uma prevalência muito maior que a esperada de SM. Um dado interessante que surge desta análise é o de que, aparentemente, o fenótipo de síndrome metabólica se torna mais evidente ao longo da vida das pacientes, e se instala na fase jovem da vida adulta. Na casuística completa publicada até a publicação deste trabalho, se considerarmos todos os 17

casos descritos na literatura (tabela 1 na introdução), dos 12 que foram avaliados antes dos 25 anos de idade, 6 ou 50% tinham um ou mais parâmetros da SM, uma prevalência muito maior que a esperada nessa faixa etária.

Quando analisamos os dados da TC abdominal que obtivemos, outro dado interessante desponta. Dados da literatura indicam que áreas de VAT acima de 131 cm^2 em algumas casuísticas ou acima de 100 cm^2 em outras, de acordo com a etnia, correlacionam-se com maior risco cardiovascular (88,89). De acordo com qualquer um dos critérios, 3 em 6 (50%) das pacientes com mutações inativadoras do *DLK1* têm acúmulo de gordura visceral acima deste ponto de corte. Apesar disso, dois familiares não afetados também têm esse indicador, e é sabido também que há diversos outros fatores que contribuem para obesidade visceral. A relação VAT/SAT é considerada indicadora de obesidade predominantemente visceral quando está acima de 0,4 (88). Segundo esse critério, portanto, 2 das 6 pacientes (33%) têm obesidade mais visceral. Isso sugere que as pacientes com deficiência do DLK1 tenham uma menor restrição à expansão do tecido adiposo, ou seja, maior facilidade em estocar gordura, dada a perda deste fator anti-adipogênico. Este fator não é absolutamente suficiente para levar a obesidade em 100% dos casos desde o nascimento, na realidade as pacientes têm peso ao nascimento normal (nem aumentado, nem diminuído como em camundongos), mas favoreceria uma maior proporção de gordura corporal quando em ambiente permissivo.

Além disso, o estudo de células vivas das pacientes, comparadas a controles, aponta que o metabolismo celular parece estar afetado. As pacientes com mutação do *DLK1* têm maior estresse oxidativo mitocondrial, maior expansão de adipócitos em meios de cultura. Além disso, o consumo de oxigênio é menor e há mais metabolismo anaeróbio celular, gerando em maior acidificação extracelular.

Em conclusão, desde a descrição da associação de mutações do *DLK1* com PPC e SM surgiram casos descritos em diversos países. Embora seja uma causa rara de PPC, o *DLK1* é um interessante modelo de adipogênese acelerada. A identificação dos riscos metabólicos de forma mais precoce permitirá o acompanhamento mais seguro das pessoas afetadas. Além disso, o potencial papel terapêutico do DLK1 ainda não foi estudado e pode ser uma via interessante de futuras pesquisas. Este trabalho abriu novas fronteiras para a compreensão da complexa correlação entre fatores genéticos, puberdade e metabolismo.

6. CONCLUSÕES FINAIS

1- Descrevemos alterações metabólicas significativas em pacientes com mutações inativadoras do gene DLK1 na fase adulta.

2- O padrão das concentrações séricas de DLK1 em pacientes saudáveis variou de acordo com o estadiamento puberal, com redução a partir do Tanner II em meninas e do Tanner III em meninos. Em pacientes com mutações inativadoras do DLK1, as concentrações séricas da proteína foram não detectáveis.

3- A deficiência de DLK1 levou à maior diferenciação de células progenitoras em adipócitos maduros, como demonstrado pela presença de maiores gotículas de gordura *in vitro*.

4- A função mitocondrial de leucócitos em pacientes com mutações do *DLK1* foi prejudicada, com evidências de maior estresse oxidativo. O padrão de ativação de macrófagos nesses pacientes foi mais direcionado às características inflamatórias.

ANEXOS

Anexo 1 - Marcadores utilizados para citometria de fluxo

Marcador	Alvo
FITC	CD64
PerCP	CD209
PE	CD16
PECy7	HLA-DR
APC	CD86
BV510	LD

Anexo 2 - Protocolo de diferenciação de células 3T3-L1

Etapa	Procedimentos
Preparação do meio	-1.: células 90% confluentes DMEM + 10%FBS + 5ug/mL insulina
Implante das células	Implante em placas em densidade de 3×10^{-3} células/cm^2
Dia 0	DMI cocktail (DEX 1uM; IBMX 0.5mM; insulina humana regular 5ug mL);
Dia 2 ao dia 8	DMEM + 10%FBS + 5ug/mL insulina Troca a cada 2 dias
Dia 9	Coleta do sobrenadante e coração com oil red para análise

Yearbook of
Paediatric Endocrinology
2021

Editors
Ken Ong
Ze'ev Hochberg

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7.2. Genotype-phenotype correlations in central precocious puberty caused by *MKRN3* mutations

Seraphim CE, Canton APM, Montenegro L, Piovesan MR, Macedo DB, Cunha M, Guimaraes A, Ramos CO, Benedetti AFF, de Castro Leal A, Gagliardi PC, Antonini SR, Gryngarten M, Arcari AJ, Abreu AP, Kaiser UB, Soriano-Guillén L, Escribano- Muñoz A, Corripio R, Labarta JI, Travieso-Suárez L, Ortiz-Cabrera NV, Argente J, Mendonca BB, Brito VN, Latronico AC

In brief: This paper describes the clinical and hormonal features of a large cohort of patients with central precocious puberty (CPP) caused by mutations in *MKRN3*. The authors found that phenotypic features of patients with *MKRN3* mutations is similar to those with idiopathic CPP.

Comment: Inactivating mutations in *MKRN3* were first identified in 2013 in families presenting with CPP (1). Later studies identified *MKRN3*, a maternally imprinted gene encoding the makorin RING-finger protein-3, as an essential inhibitory component of the gene network governing puberty (2-4). *MKRN3* mutations that cause CPP include frameshift, stop gain and missense mutations affecting the coding or gene promoter region (5). *MKRN3* mutation is currently the most common monogenic cause of familial CPP (5).

In this study, a multi-ethnic cohort of 716 patients with familial or idiopathic CPP was screened for *MKRN3* mutations using Sanger sequencing and compared to 156 Brazilian girls with idiopathic CPP. Forty-five girls and 26 boys from 36 unrelated families presented loss-of-function mutations. Among the patients with *MKRN3* mutations, first pubertal signs occurred at 6.2 ± 1.2 years in girls and 7.1 ± 1.5 years in boys, and bone age advancement was 2.0 ± 1.6 and 1.8 ± 1.3 years, respectively. Basal LH levels were 1.9 ± 1.8 IU/l in girls and 1.6 ± 1.2 IU/l in boys. The patients with severe mutations (frameshift mutations, stop gain variants or promoter region deletions) had greater bone age advancement and higher basal LH levels than patients with missense mutations. Girls with *MKRN3* mutations had a shorter delay between puberty onset and first evaluation than girls with idiopathic CPP, independently of family history.

Overall, this study suggests that the phenotypic presentation of patients with *MKRN3* mutations is similar to those with idiopathic CPP. They present a shorter time between pubertal onset and first evaluation, as well as higher FSH levels. Severe mutations (as predicted by *in silico* analysis) lead to greater bone age advancement and higher basal LH levels.

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Anexo 4 - Lista de artigos publicados durante o doutoramento

Ano	Artigo	IF	Citações
2019	"Pioneering studies on monogenic central precocious puberty" *Canton APM,* **Seraphim CE***, Brito VN, Latronico AC* *Arch Endocrinol Metab. 2019 Aug 22;63(4):438-444*	2,03	39
2020	"Delta-like 1 homolog genetics and its emerging role in human puberty" **CE Seraphim***, J Argente, AC Latronico* *Current Opinion in Endocrine and Metabolic Research 2020. 14, 22-28*	1,9	
	"Outcomes of Patients with Central Precocious Puberty Due to Loss-of-Function Mutations in the MKRN3 Gene after Treatment with Gonadotropin-Releasing Hormone Analog." *Ramos CO, Macedo DB, (...),* **Seraphim CE***, (...), Latronico AC, Brito VN* *Neuroendocrinology. 2020;110(7-8):705-713*	5,14	18
	"Novel genetic and biochemical findings of *DLK1* in children with central precocious puberty: a Brazilian–Spanish Study" *Montenegro L, Labarta JI, (...) Seraphim* **CE***, (...) Latronico AC, Argente J* *J Clin Endocrinol Metab. 2020 Oct 1;105(10): dgaa461*	6,13	29
2021	"Anthropometric, metabolic, and reproductive outcomes of patients with central precocious puberty treated with leuprorelin acetate 3-month depot (11.25 mg)" *CO Ramos, APM Canton,* **CE Seraphim***, AG Faria, FR Tinano* *J Pediatric Endocrinology and Metabolism 2021; 34 (11), 1371-1377*	1,52	3
	"Genotype–Phenotype Correlations in Central Precocious Puberty Caused by MKRN3 Mutations" **CE Seraphim,** *APM Canton, L Montenegro, MR Piovesan, DB Macedo, ...* *J Clin Endocrinol Metab. 2021 Mar 25;106(4):1041-1050*	6,13	36
2022	"Precocious sexual maturation: Unravelling the mechanisms of pubertal onset through clinical observations." *L Soriano-Guillén, M Tena-Sempere,* **CE Seraphim***, AC Latronico, ...* *Journal of neuroendocrinology 2022; 34 (2), e12979*	3,87	9
2023	"The congenital and acquired mechanisms implicated in the etiology of central precocious puberty." *VN Brito, APM Canton,* **CE Seraphim***, AP Abreu, DB Macedo, ...* *Endocr Rev. 2023 Mar 4;44(2):193-221*	25,26	13
	"Clinical and genetic characterization of familial central precocious puberty" *Tinano FR, Canton APM, (...) Seraphim* **CE,** *(...) Latronico AC* *J Clin Endocrinol Metab. 2023 Jun 16;108(7):1758-1767*	6,13	5
	"Rare variants in the MECP2 gene in girls with central precocious puberty: a translational cohort study" Canton APM, Tinano FR, (…), **Seraphim CE**, (…) Latronico AC Lancet Diabetes Endocrinol. 2023 Aug;11(8):545-554.	44,5	1
	"Familial central precocious puberty due to DLK1 deficiency: novel genetic findings and relevance of serum DLK1 levels" *L Montenegro*,* **CE Seraphim****, F Tinano, M Piovesan, APM Canton, ...* *European Journal of Endocrinology 2023; 189 (3), 422-428* **BOTH AUTHORS CONTRIBUTED EQUALLY AS FIRST AUTHORS*	6,56	1

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