Circulating MKRN3 Levels Decline Prior to Pubertal Onset and Through Puberty: A Longitudinal Study of Healthy Girls

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Context: Puberty is initiated by a complex interaction of suppressing and stimulating factors. Genetic studies of familial central precocious puberty have suggested makorin ring finger protein 3 (MKRN3) as a major inhibitor of GnRH secretion during childhood. Furthermore, genetic variation near MKRN3 (rs12148769) affects age at menarche in healthy girls.

Objective: The purpose of this study was to evaluate whether serum levels of MKRN3 declined before pubertal onset in healthy girls.

Design: This was a population-based longitudinal study of healthy Danish girls and a cohort study of early maturing girls.

Setting: The study was performed in the general community and in a tertiary referral center for pediatric endocrinology.

Patients or Other Participants: Healthy girls (n = 38) aged 9.3 years (range, 5.9–11.3 years) at baseline and followed for 6.0 years (2.7–7.6 years) (2006–2014) with blood sampling every 6 months and early maturing girls (n = 13) with breast development at 8.3 years of age were included.

Main Outcome Measures: Serum levels of MKRN3 were measured in 354 samples (median, 9 per girl; range, 2–14 per girl), and genotyping of variants near MKRN3 (rs12148769 and rs12439354) was performed.

Results: MKRN3 concentrations declined preceding pubertal onset; the geometric mean (95% confidence interval) 3 years before pubertal onset vs the last visit before pubertal onset was 304 pg/mL (264–350 pg/mL) vs 257 pg/mL (243–273 pg/mL), corresponding to a reduction of 15% (1–27%) (P = .033). In prepubertal girls, circulating MKRN3 correlated negatively with gonadotropin levels: for FSH, \( r = -0.262 \) (\( P = .015 \)) and for LH, \( r = -0.226 \) (\( P = .037 \)). After adjustment, MKRN3 levels were lower in early maturing girls than in age-matched prepubertal girls: 171 pg/mL (25–333 pg/mL) vs 262 pg/mL (94–624 pg/mL) (\( P = .051 \)). Genetic variants near MKRN3 did not correlate with serum levels of MKRN3.

Conclusions: Declining levels of circulating MKRN3 preceded pubertal onset. The negative correlation between MKRN3 and gonadotropins further supports MKRN3 as a major regulator of hypothalamic GnRH secretion during childhood. Undetectable or low MKRN3 levels were observed in a subgroup of patients with early onset of puberty. (J Clin Endocrinol Metab 100: 1920–1926, 2015)
Puberty is initiated by reactivation of the hypothalamic GnRH pulse generator. The transient activation of the hypothalamic-pituitary-gonadal (HPG) axis during infancy is followed by a quiescent period in childhood (1). Withdrawal of hypothalamic inhibition reactivates GnRH secretion, which initiates puberty (2). Despite intensive research, little is known about the specific factors responsible for childhood inhibition of hypothalamic activity essential for timing of pubertal onset (3).

Much attention was therefore focused on the study conducted by Abreu et al (4) describing mutations in the gene encoding makorin ring finger protein 3 (MKRN3) in 5 of 15 families with central precocious puberty (CPP). This was the first report of a deleterious mutation associated with CPP, suggesting MKRN3 as a major inhibitor of hypothalamic GnRH secretion during childhood. In support, the authors reported declining mRNA expression of hypothalamic Mkrn3 preceding pubertal onset in mice of both sexes. Several reports of MKRN3 mutations in familial CPP have been published since then (5–8).

MKRN3 is an intronless gene located on chromosome 15q11.2 in the Prader-Willi syndrome critical region. The maternal allele is imprinted (silenced), and mutations are therefore paternally inherited (9). The precise mechanism of MKRN3 remains to be elucidated; however, the zinc finger structure indicates properties such as transcriptional modulation of DNA or RNA and/or posttranslational modification of proteins (10). Recent genome-wide associations studies have found that the paternal allele of a specific single nucleotide polymorphism (SNP) found in an intergenic region near MKRN3 and MAGEL2 (rs12148769, G>A) affects age at menarche in healthy girls (11).

To our knowledge, the serum levels of MKRN3 have never been reported in animal models or humans. We measured circulating levels of MKRN3 in healthy girls followed longitudinally through puberty as well as in early maturing girls. Our findings confirmed the main hypothesis of declining MKRN3 levels preceding pubertal onset.

### Subjects and Methods

#### Healthy girls

The COPENHAGEN Puberty Study is a combined cross-sectional and ongoing longitudinal population-based cohort study of healthy Danish children and adolescents. The primary purpose for conducting the study was to establish the time of pubertal onset in a contemporary cohort of Danish children (12, 13). Predefined secondary outcomes were to establish normative data for anthropometry and hormone levels during childhood and adolescence. All pupils (n = 6203) at 10 randomly selected schools in the Copenhagen area were invited to participate in the cross-sectional study (2006–2008). All schools were situated in areas of Copenhagen traditionally representing the social middle class. The overall participation rate was 30%, ranging from 19% to 40%. All participants at 2 schools with high participation rates were invited to continue in the ongoing longitudinal follow-up study.

Blood samples were drawn, and a thorough clinical examination was performed, including staging of breast development by palpation (B1–B5) according to the Tanner classification (14). Pubertal onset was defined as having Tanner breast stage 2 (B2) or more, as described previously in detail (12). The age of pubertal onset was approximated using the date exactly between the 2 visits at which the girl advanced from B1 to B2 (or more).

From a total of 108 girls participating in the longitudinal part of the COPENHAGEN Puberty Study, girls were excluded from the present dataset for the following reasons: blood samples were only drawn before or after pubertal onset (n = 43), the age at pubertal onset was not well defined (examinations with Tanner breast stage B2 were followed by examinations with B1) (n = 10), one or both parents originated from a non-European country (n = 16), and previous cytostatic treatment (n = 1). The remaining 38 healthy Caucasian girls with available blood samples before and after age at pubertal onset were included in the present study. The girls had no history of gynecological or cerebral diseases. They were followed for an average of 6.0 years (range, 2.7–7.6 years). Median (range) age at baseline was 9.3 years (5.9–11.3 years). Serum concentrations of MKRN3 were measured in a total of 354 samples (individual samples per girl: median, 9; range, 2–14). FSH and LH levels were measured in 240 samples from 38 girls (86 samples from 3.5 prepubertal girls). Data on gonadotropins have been published previously (15).

#### Early maturing girls

MKRN3 was measured in early maturing girls participating in a prospective clinical study. A detailed description of the patients is available in the original publication (16). In short, patients referred with an onset of breast development before the age of 9 years were recruited consecutively from our outpatient clinic at the Department of Growth and Reproduction, Copenhagen University Hospital, from May 2008 to September 2009. All patients presented with breast budding as the first sign of puberty and all were premenarcheal. Centrally activated puberty was defined as a peak LH level of ≥ 5 IU/L in response to the rapid-acting GnRH agonist (0.1 mg of Relefact LHRH; Sanofi-Aventis) (17). CPP was diagnosed if breast development started before age 8 years. Two girls with breast development at 8.3 years were characterized as having early puberty. Brain magnetic resonance imaging was nonpathological.

We measured MKRN3 in baseline samples (before initiation of leuprolide acetate treatment) from 13 of 15 girls (samples from 2 girls were not available). The median (range) age at pubertal onset was 7.6 years (7.2–8.3 years). The age at the baseline examination was 8.4 years (7.5–9.2 years). The bone age at baseline was 9.6 (7.7–10.8).

#### Reproductive hormone assays

All blood samples were drawn between 8:00 AM and 1:00 PM from an antecubital vein, clotted, and centrifuged, and serum was stored at −20°C until hormone analyses were performed. Blood samples were analyzed after a maximum of 8 years of storage in the freezer at −20°C. All samples were analyzed in the...
same laboratory blinded for the technician for age and pubertal stage.

Serum MKRN3 concentrations were determined using the commercially available human makorin ring finger protein 3 ELISA (MyBioSource) with a detection limit of 2.5 pg/mL. Intra- and interassay coefficients of variation (CVs) listed by the manufacturer were <8% and <10%, respectively. In our hands, the intra-assay CVs (SD/mean) were 5.4% at 201 pg/mL and 5.1% at 410 pg/mL, respectively, and the interassay CV was 9.7% at 288 pg/mL. All samples from a given girl were analyzed on the same plate.

On the plate including early maturing girls, the control was 16% higher than the average of controls on the remaining plates. In an additional analysis, we adjusted all results with a plate-specific correction factor: mean of controls (reference plate)/mean of controls (adjusted plate).

Serum levels of FSH and LH were measured by time-resolved immunofluorometric assays (Delfia) with detection limits of 0.06 and 0.05 IU/L, respectively. Intra- and interassay CVs were <5%. LH levels below the detection limit were assigned the value of 0.025 IU/L (0.5 times the detection limit).

Genotyping
Peripheral blood (0.2 mL of EDTA preserved) was used for isolation of genomic DNA using the QuickGene-810 Nucleic Acid Isolation System (Fujifilm, Life Science Products) and quantified on a NanoDrop ND-1000 spectrophotometer (Saveen Werner). Purified DNA was available for 34 healthy girls with prepubertal MKRN3 measurements as well as 12 early maturing girls (DNA was limited in 1 patient, allowing analysis of only 1 SNP).

The SNPs were analyzed using KASP SNP genotyping assays (LGC Genomics), which facilitate biallelic discrimination through a competitive PCR and incorporation of a fluorescent resonance energy transfer quencher cassette. KASP genotyping assays were designed by LGC Genomics toward the following sequences: rs12148769, TTATGCATTGTCAA[R]CTTCAAA-GGCCACATAA; and rs12439354, TAGTTATTATTTCCTCA [R]GATGACTGCCAG. rs12148769 is well known from association studies of menarche (11), whereas rs12439354 was found from the potentially functional SNP search engine (18) to potentially affect a peroxisome proliferator-activated receptor γ/retninoid X receptor α binding site in the promoter of MKRN3. A standard touch-down PCR program, as advised by the manufacturers, was used to discriminate alleles.

Statistical analyses
To evaluate the individual fluctuation of MKRN3, we depicted individual levels according to time from pubertal onset. The girls were divided in tertiles based on means of prepubertal MKRN3. In addition, we assessed the intraindividual CVs.

To further evaluate the progress of MKRN3 levels as a function of time from pubertal onset, we used a variance component model allowing each girl to have her own general MKRN3 level (15). The time from B2 (numeric variable) was grouped into a categorized variable, ie, −3 years: samples < −2.5 years from B2; −2.5 ≤ −2 years < −1.5; −1.5 ≤ −1 year < −0.5; −0.5 ≤ −0.5 year < 0; 0 ≤ 0.3 year < 0.5; 0.5 ≤ 1 year < 1.5; 1.5 ≤ 2 years < 2.5; 2.5 ≤ 3 years < 3.5; 3.5 ≤ 4 years < 4.5; 4.5 ≤ 5 years < 5.5; 5.5 ≤ 6 years < 6.5. In case of multiple MKRN3 values per girl in a given time interval, the mean MKRN3 concentration was used. To compensate for a skewed distribution of MKRN3, we transformed MKRN3 values with the natural logarithm before analysis.

To assess the correlations between (1) MKRN3 and gonadotropin levels in samples from prepubertal girls and (2) prepubertal MKRN3 (mean of individual prepubertal values) and age at pubertal onset, the Spearman correlation was used. The association between prepubertal MKRN3 levels and pubertal onset was further explored by comparing age at pubertal onset in girls from different MKRN3 tertiles using the Mann-Whitney U test.

To evaluate whether MKRN3 levels were affected in patients with CPP and early maturing girls (n = 13), we compared the MKRN3 levels with those of aged-matched prepubertal girls (n = 21) using the Mann-Whitney U test. If more samples were available from each girl, we used the mean of individual measurements. In an additional analysis, we adjusted all samples with a plate-specific correction factor.

To evaluate whether MKRN3 SNPs affected the circulating levels of MKRN3, we used the Mann-Whitney U test (2 groups) or the Kruskall-Wallis test (3 groups).

Ethical considerations
The Copenhagen Puberty Study (ClinicalTrials.gov identifier NCT01411527) and the study of early maturing girls were approved by the local ethics committee (KF 01 282214, V200.1996/90, KF 01 282214, and KF 11 2006-2033) and the Danish Data Protection Agency (2010-41-5042). All participants and their parents gave informed consent.

Results
Circulating MKRN3 varied substantially between individuals; the median (range) of all samples was 244 pg/mL (<25–653 pg/mL). Each girl seemed to maintain her relative MKRN3 level (Figure 1); the median (range) of intra-individual CVs was 16.6% (6.3–33.4%).

MKRN3 levels declined preceding pubertal onset; the geometric mean (95% confidence interval) 3 years before pubertal onset vs the last visit before pubertal onset was 304 pg/mL (264–350 pg/mL) vs 257 pg/mL (243–273 pg/mL), respectively, corresponding to a reduction in MKRN3 of 15% (1–27%), P = .037 (Figure 2). The MKRN3 levels declined continuously as puberty progressed (Figure 2).

When 1 sample per girl (mean of individual values) was included, the prepubertal MKRN3 levels (n = 35) did not correlate with circulating FSH (r = −0.257, P = .137) or LH (r = −0.261, P = .129). When all available samples from prepubertal girls (n = 86 from 35 girls) were included, the circulating MKRN3 concentrations correlated negatively with the gonadotropin levels (ie, for FSH, r = −0.262, P = .015 and for LH, r = −0.226, P = .037) (Figure 3).
There were no linear correlations between prepubertal MKRN3 concentrations (mean of individual prepubertal values) and age at pubertal onset ($r = 0.069$, $P = .692$; data not shown). Age at pubertal onset according to tertiles of prepubertal MKRN3 indicated a nonlinear association. Girls with the highest prepubertal MKRN3 (tertile 1) entered puberty later than girls with medium MKRN3 (tertile 2); the median age at pubertal onset was 10.7 years (range, 9.2–12.1 years) vs 9.7 years (8.8–11.7 years) ($P = .019$). There was no difference in age at pubertal onset between girls with medium and low prepubertal MKRN3 (tertile 2 vs tertile 3): 9.7 years (8.8–11.7 years) vs 10.6 years (9.2–12.6 years) ($P = .106$; not shown).

In a crude analysis, MKRN3 levels were comparable between patients with CPP and early maturing girls compared with age-matched prepubertal girls: 203 pg/mL (<25–395 pg/mL) vs 255 pg/mL (101–620 pg/mL) ($P = .257$; Figure 1). After adjustment of all samples with plate-specific correction factors, MKRN3 levels were lower in early maturing girls: 171 pg/mL (<25–333 pg/mL) vs 262 pg/mL (94–624 pg/mL) ($P = .051$; not shown).

Importantly, 4 of 13 early maturing girls (31%) had MKRN3 levels around or below the fifth percentile of that for age-matched healthy prepubertal girls. One patient with pubertal onset at age 7.4 years had undetectable concentrations of MKRN3 in serum (Figure 1).

### MKRN3 SNPs

The girls had the following allele distributions (healthy girls + early maturing girls): for MKRN3 (rs12148769, G>A): GG, 23+10; GA, 10+1; AA, 1+0; and minor allele frequency, 14%; for MKRN3 (rs12439354, A>G): AA, 26+7; AG, 8+4; GG, 0+1; and minor allele frequency, 15%. Distributions were consistent with Hardy-Weinberg equilibrium (Pearson $\chi^2 = 0.006$, $P = .941$; and $\chi^2 = 0.005$, $P = .941$, respectively).

In healthy girls, neither of the SNPs was associated with serum levels of MKRN3 (individual mean of prepubertal MKRN3): for MKRN3 (rs12148769, G>A): GG (n = 23), 267 pg/mL (84–471 pg/mL) vs GA (n = 10), 267 pg/mL (91–581 pg/mL) vs AA (n = 1), 561 pg/mL ($P = .033$).
.280; Kruskall-Wallis); for MKRN3 (rs12439354, A>G): AA (n = 26), 268 pg/mL (91–581 pg/mL) vs AG (n = 8), 265 pg/mL (84–381 pg/mL) (P = .655; Mann-Whitney U).

No associations between MKRN3 genotypes and serum levels were found in early maturing girls: for MKRN3 (rs12148769, G>A): GG (n = 10), 182 pg/mL (<25–395 pg/mL) vs GA (n = 1), 355 pg/mL (P = .343); for MKRN3 (rs12439354, A>G): AA (n = 7), 196 pg/mL (<25–388 pg/mL) vs AG (n = 4), 195 pg/mL (48–395 pg/mL) vs GG (n = 1), 344 pg/mL (P = .760).

Discussion

This is the first study describing circulating MKRN3 in serum. The decline of MKRN3 preceding puberty as well as the negative correlation with gonadotropins supports MKRN3 as a major inhibitor of hypothalamic GnRH secretion during childhood. Furthermore, undetectable or low levels of MKRN3 were observed in a subgroup of patients with idiopathic CPP.

Puberty is initiated by reactivation of the HPG axis. Genetic analyses of patients with delayed puberty and hypogonadotropic hypogonadism have revealed several activators essential for hypothalamic GnRH secretion, eg, kisspeptin and neurokinin B (19–22). Although excitatory input is essential, withdrawal of hypothalamic inhibition seems to be a prerequisite for hypothalamic reactivation initiating pubertal onset (3). The study of MKRN3 mutations in familial CPP of Abreu et al (4) was the first to suggest MKRN3 as a major inhibitor of hypothalamic activity during childhood. Our findings of decreasing levels of circulating MKRN3 are in line with the decrease in hypothalamic Mkrn3 expression (mRNA) preceding pubertal onset in mice (4). However, the profile of the expression levels indicated a more abrupt termination of MKRN3 than the gradual decline in serum levels, which we observed in the present study. Supporting inhibition of GnRH secretion, serum levels of MKRN3 were negatively correlated with circulating FSH and LH levels in samples from prepubertal girls. Although the specific mechanism of MKRN3 remains to be elucidated, the zinc finger structure and presumed ubiquitin ligase properties indicate that MKRN3 regulates cellular processes such as posttranslational modification of proteins or epigenetic regulation of transcription (10). Our findings therefore support the assumption put forward by Ojeda and Lomniczi (2) that MKRN3 inhibits downstream activators of hypothalamic GnRH secretion, such as KISS1.

The substantial interindividual variations in circulating MKRN3 indicate that there is no common threshold for circulating MKRN3 to initiate puberty. Despite declining MKRN3, each girl maintained her relative level. This finding may suggest the existence of an individual set point at which the relative decline in serum levels of MKRN3 is important for the withdrawal of hypothalamic inhibition. Genetic variations in MKRN3 reported to affect age at menarche (11) did not seem to be associated with the serum levels of MKRN3 either in healthy girls or in early maturing girls. However, the number of girls in this study is not large enough to draw a firm conclusion on the effect of the 2 MKRN3 SNPs, and we do not have information about which of the alleles were paternally inherited. In particular, we lack power in the group of girls being homozygous for the minor alleles, and we are not able to evaluate combinations of the 2 SNPs. The girl with CPP and undetectable MKRN3 was homozygous for the major alleles in both SNPs; however, this does not exclude other deleterious mutations in her MKRN3 gene.

We did not observe a linear correlation between prepubertal MKRN3 concentrations and age at pubertal on-
set in healthy girls, but we cannot exclude the possibility that MKRN3 affects the timing of puberty. First of all, we observed that girls with the highest tertile of prepubertal MKRN3 entered puberty later than the girls with medium MKRN3. Second, in adjusted analyses, MKRN3 levels were lower in early maturing girls than in age-matched prepubertal girls, and, interestingly, a girl with very early onset of puberty (7.4 years) had undetectable concentrations of MKRN3 in serum. Thus, low levels of circulating MKRN3 may cause CPP in a subgroup of patients.

The substantial interindividual variation in MKRN3 concentrations as well as the complex interpretation of associations between MKRN3 concentrations and age at pubertal onset emphasize the fact that multiple regulators of puberty exist (eg, genetic, epigenetic, metabolic, and environmental). This is also reflected by the high number and the great diversion of genetic polymorphisms affecting the timing of pubertal onset, eg, genes encoding microRNA-binding proteins (LIN28B) (23) and genes affecting FSH signaling (FSHB and FSHR) (24).

The continuous decline of MKRN3 before and after the time of breast development suggests that the withdrawal of hypothalamic inhibition is a gradual transition lasting for years. The graduate increase in gonadotropins through pubertal development may reflect this process, indicating that the release of the brake initiating pubertal onset is a slow transition and not a sudden termination. This is supported by gradually increasing circulating levels of estradiol, increasing uterus volume and a high prevalence of mature ovarian follicles in girls before pubertal onset (25–28).

The healthy girls in the present study represent the background population of Danish girls. Pubertal onset was evaluated by the gold standard, which includes physical examination of breast development, and the frequent examinations enabled us to determine the age at pubertal onset with high accuracy. Circulating MKRN3 levels have not been reported previously, and therefore external validation of the specificity of the assay is not available. However, according to the manufacturer, the assay is specific for human MKRN3 without any cross-reactions. The isolated phenotype of premature pubertal onset in patients with deleterious MKRN3 mutations supports the role of MKRN3 being at the hypothalamic level.

In conclusion, declining levels of circulating MKRN3 preceded pubertal onset and concentrations decreased further as puberty progressed. The negative correlation between MKRN3 and gonadotropins in prepubertal girls further supports MKRN3 as a major regulator of HPG activity during childhood. In a subgroup of patients with CPP, MKRN3 was low or undetectable. This may imply a causal relationship.

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