Pubertal Onset in Girls is Strongly Influenced by Genetic Variation Affecting FSH Action

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Age at pubertal onset varies substantially in healthy girls. Although genetic factors are responsible for more than half of the phenotypic variation, only a small part has been attributed to specific genetic polymorphisms identified so far. Follicle-stimulating hormone (FSH) stimulates ovarian follicle maturation and estradiol synthesis which is responsible for breast development. We assessed the effect of three polymorphisms influencing FSH action on age at breast development in a population-based cohort of 964 healthy girls. Girls homozygous for FSHR -29AA (reduced FSH receptor expression) entered puberty 7.4 (2.5–12.4) months later than carriers of the common variants FSHR -29GG, p=0.003. To our knowledge, this is the strongest genetic effect on age at pubertal onset in girls published to date.

Pubertal onset varies substantially among healthy girls and only part of the variation can be explained by racial differences. Timing of puberty has received considerable attention due to the associations with numerous adverse conditions later in life, e.g. behavioural disorders, breast cancer, obesity, metabolic syndrome, and cardiovascular disease. Although genetic factors seem responsible for more than half of the phenotypic variation, knowledge of specific regulators of pubertal onset is limited. Abnormal pubertal timing can be caused by rare mutations: e.g. MKRN3, ESR1, KISS1, KISS1R whereas common genetic variations in candidate loci (KISS1, KISS1R, LIN28B, LIN28A) do not seem to be involved in idiopathic central precocious puberty. Additionally, large genome-wide association (GWA) studies have drawn attention to single nucleotide polymorphisms (SNP) affecting pubertal timing. Nevertheless, only approximately 15% of the variance in age at menarche can be explained by genetic variations identified in GWA studies.

The majority of clinical studies of pubertal timing are based on recall of age at menarche which is a late pubertal event occurring in average three years after onset of breast development. Growth of breast tissue is the first clinical sign of the pubertal reactivation of the hypothalamic-pituitary-gonadal axis resulting in ovarian estradiol production. Assessment of breast development is considered the golden standard when evaluating pubertal onset and progression in girls.

Follicle-stimulating hormone (FSH) is essential for female reproductive maturation. FSH induces ovarian follicle growth and aromatase activity, hence, estradiol synthesis responsible for breast development, and genetic variants in FSHB and FSHR have been reported as modulators of FSH action.

FSHB -211G>T is located in the promoter of the gene encoding the FSH beta subunit. Reduced FSH production has been suggested to carriers of FSHB -211TT vs. GG due to altered affinity of the LHX3 transcription factor binding element.

FSHR 2039A>G is located in the coding region of the intracellular domain of the FSH receptor. Carriers of FSHR 2039GG have slower intracellular cAMP production (assessed in human granulosa cells) and need more exogenous FSH for sufficient ovarian stimulation than FSHR 2039AA homozygotes.

FSHR -29G>A is located in the promoter region of the gene encoding the FSH receptor. In in-vitro fertilisation patients, the FSH dose had to be increased 83% in order to induce follicular growth in carriers of FSHR -29AA vs. GG. Despite increased exogenous FSH, the numbers of mature and retrieved oocytes were reduced 40% and 34%, respectively. The impaired ovarian FSH response in women with FSHR -29AA was supported by DNA topography and expression analyses, which indicated that the A allele was less accessible for binding of tran-
scription factors compared with the G allele. In support, FSH receptor expression at both mRNA and protein level was reduced in granulosa cells from carriers of FSHR -29AA vs. GG\(^{3,26}\).

Recently, we observed that FSHR -211G>T and FSHR 2039A>G may influence age at pubertal onset in girls, but due to the relatively small sample size of 78 girls, no firm conclusions could be drawn from the study\(^2\). We have since genotyped a large cohort of 964 healthy Danish girls as well as female patients with idiopathic delayed puberty, including evaluation of the FSHR -29G>A SNP.

We report how the three polymorphisms affect age at pubertal onset evaluated by breast development, revealing a remarkably strong effect of FSHR -29G>A.

**Results**

**Healthy girls.** At evaluation, the mean age of the entire study population was 10.8 (9.6–12.1) years (+/− 1SD), and the mean age at pubertal onset was 10.0 (9.9–10.2) years (95% CI).

The girls had the following allele distributions: FSHR -211G>T (GG 650, GT 290, TT 22, minor allele frequency (MAF) 17%), FSHR -29G>A (GG 512, GA 368, AA 74, MAF 27%), and FSHR 2039A>G (AA 242, AG 504, GG 218, MAF 49%). Distributions were consistent with Hardy-Weinberg equilibrium (Pearson’s \(\chi^2\) = 2.47, \(p = 0.116\); \(\chi^2\) = 0.48, \(p = 0.488\); and \(\chi^2\) = 2.07, \(p = 0.151\), respectively).

Age at pubertal onset according to the presence of genetic variants of FSHR -29G>A, FSHB -211G>T, and FSHR 2039A>G is listed in Table 1. We observed a strong effect of the SNP in the FSH receptor promoter (FSHR -29G>A). Girls homozygous for the minor allele (FSHR -29AA, \(n = 74\)) entered puberty 7.4 (2.5–12.4) months later than carriers of the more common variants FSHR -29GG+GA, 10.6 (10.2–11.0) vs. 10.0 (9.8–10.1), \(p = 0.003\) (Figure 1A). BMI was not affected by genotypes (Table 1), and adjusting for BMI did not alter the effect of FSHR -29AA vs. GG+GA, \(p = 0.004\) (data not shown).

The additive model indicated a co-dominant effect of the alleles in which the heterozygotes exhibited intermediate levels; the age at pubertal onset increased 2.4 (0.2–4.5) months per A allele, \(p = 0.031\) (Table 1).

Girls with the rare FSHB -211TT genotype (\(n = 22\)) seemed to enter puberty later than carriers of FSHB -211GG and GT, but the difference did not reach significant levels in any of the models. Even after adjusting for FSHR -29G>A, there was no clear effect of FSHB -211GG+GT vs. TT, \(p = 0.129\) (data not shown). FSHR 2039A>G did not seem to influence age at pubertal onset (Table 1).

In a combined model of FSHR -29G>A and FSHR -211G>T, age at pubertal onset increased with increasing number of minor alleles across the genotypes; i.e. 1.9 (0.2–3.6) months per minor allele, \(p = 0.025\) (Figure 1B).

We estimated that 2.2% and 2.9% of the variance in age at pubertal onset could be explained by FSHR -29G>A alone and in combination with FSHR -211G>T, respectively.

**Patients with delayed puberty.** Age at breast development for the patients with delayed puberty was 13.8 years (13.0–17.0). Puberty was induced by exogenous estradiol in four patients at age 13.0, 13.1, 14.5, and 17.0 years. The remaining patients entered puberty spontaneously. The SNPs causing later pubertal onset in healthy girls (FSHR -29AA and FSHB -211TT) were significantly more prevalent in patients with idiopathic delayed puberty compared with healthy girls (5/18 patients = 28% vs. 96/964 healthy girls = 10%, \(p = 0.014\)). Distribution of genotypes are shown in Figure 2.

**Discussion**

This large population-based study of 964 healthy Danish girls revealed that breast development (marking the onset of puberty) occurred on average 7.4 months later in carriers of FSHR -29AA genotype compared with the common genotype variants FSHR -29GG+GA. Based on a combined model of FSHR -29G>A and FSHB -211G>T, the age at pubertal onset increased with the number of minor alleles across the genotypes. To our knowledge, a stronger genetic influence on pubertal onset in girls has not previously been reported.

Considerable effort has been invested in mapping genetic variations responsible for pubertal timing. From a recent large-scale meta-analysis of 57 GWA studies including data from 182,416 women, 106 genomic loci associated with cellular development, body weight, and hormone pathways were proposed to affect age at menarche\(^{11}\). However, compared with our selective approach of assessing the effect of biological relevant SNPs, the effect estimates in GWA studies were limited and the strongest association was responsible for only a 1.3 months change in age at menarche per allele (LIN28B). All of the suggested loci only explained 15% of the variance in age at menarche. In this perspective, our finding of two SNPs explaining

| Table 1 | Clinical parameters of the study population stratified by genotypes |
|---------|-----------------------|
|          | Genotype |            | Additive model | Additive model a |
| **FSHR -211G-T** |          |            |               |               |
| n | 650 | 290 | 22 |               |               |
| Age (years) | 10.8 (9.6–12.1) | 10.8 (9.5–12.0) | 11.1 (10.1–12.1) | p = 0.346 | p = 0.416 |
| BMI (kg/m²) | 17.3 (15.0–19.8) | 17.3 (15.0–19.9) | 17.6 (15.8–19.6) | p = 0.560 | p = 0.816 |
| Age at pubertal onset | 10.0 (9.8–10.1) | 10.1 (9.8–10.3) | 10.7 (10.5–10.9) | p = 0.246 | p = 0.437 |
| **FSHR -29G-A** |          |            |               |               |
| n | 512 | 74 | 368 |               |               |
| Age (years) | 10.8 (9.6–12.1) | 10.8 (9.6–12.1) | 10.8 (9.6–12.1) | p = 0.736 | p = 0.898 |
| BMI (kg/m²) | 17.2 (15.1–19.5) | 17.5 (15.1–20.2) | 17.2 (14.7–20.0) | p = 0.660 | p = 0.133 |
| Age at pubertal onset (years) | 10.0 (9.8–10.1) | 10.0 (9.8–10.2) | 10.6 (10.2–11.0) | p = 0.003 | p = 0.031 |
| **FSHR 2039A>G** |          |            |               |               |
| n | 242 | 504 | 218 |               |               |
| Age (years) | 10.8 (9.6–12.1) | 10.8 (9.6–12.0) | 10.9 (9.6–12.1) | p = 0.715 | p = 0.443 |
| BMI (kg/m²) | 17.4 (15.0–20.3) | 17.2 (15.1–19.7) | 17.3 (15.2–19.7) | p = 0.960 | p = 0.816 |
| Age at pubertal onset | 10.0 (9.7–10.2) | 10.0 (9.9–10.2) | 10.1 (9.8–10.3) | p = 0.527 | p = 0.579 |

Age: mean (+/− SD), BMI: geometric mean (+/− SD), Age at pubertal onset: mean (95% CI).

In the recessive model, strong effects are expected only in minor allele homozygotes. These are compared with pooled wildtype and heterozygotes (e.g. FSHR 29GG+GA vs. AA).

Differences in ages and BMI levels between genotypes were assessed with independent samples t-test.

Differences in ages at pubertal onset between genotypes were assessed with probit analysis (categorical variable).

The additive model assumes a codominant effect of alleles in which the heterozygotes should exhibit intermediate levels, regressions are calculated over, for example, FSHR 29GG vs. GA vs. AA.

Differences in ages and BMI levels between genotypes were evaluated with One-way ANOVA.

Differences in ages at pubertal onset between genotypes were assessed with probit analysis (continuous variable).
2.9% of the variance in age at breast development is remarkable. Although we compare how SNPs affect two different milestones of pubertal development (breast development and menarche), estradiol is essential for both events. Furthermore, genes associated with age at menarche were also associated with age at breast development, e.g. LIN28B.

Noteworthy, FSHR -29G>A and FSHB -211G>T were not included in commercial genotyping platforms (e.g. Affymetrix and Illumina) commonly used in GWA studies (http://ncbi.nlm.nih.gov/SNP), which is probably why they have not been identified previously. An indirect way to confirm the observed effect would be to review GWA studies concerning the effect of other SNPs inherited with FSHR -29G>A and FSHB -211G>T (tagSNPs).

However, no such tagSNPs exist (http://www.ensembl.org/Homo_sapiens/Variation).

FSH is essential for female reproductive maturation, and our findings are therefore supported by a plausible biological mechanism. Female FSHB and FSHR knock-out mice and women with loss-of-function mutations in these specific genes present with primary amenorrhea and sterility due to disrupted follicle maturation.

In our study, the main effect on pubertal onset seemed to be driven by genetic variation in the promoter of the gene encoding the FSH receptor, FSHR -29G>A. This was first described by Wunsch and colleagues who did not observe effect on promoter activity in in vitro studies of nonhuman granulosa- and sertoli-cell lines. However, in vitro studies of human granulosa cell lines as well as cohort studies of IVF patients suggested reduced FSH receptor expression in carriers of FSHR -29AA vs. GG.

As in our study, intermediate effect was found in heterozygotes (FSHR -29AG). The effect of FSHB -211TT (reduced FSH production) seemed to be even stronger than FSHR -29AA. Despite our fairly large sample size, we acknowledge that the FSHB -211TT subgroup is small due to the very low minor allele frequency, and the lack of statistical significant effect of the FSHB -211TT (when evaluated separately) may therefore be due to a type II error. The combined model suggests that both the FSHR -29A allele and the FSHB -211T allele contribute to postpone pubertal onset.

Our finding of higher prevalence of FSHR -29AA and FSHB -211TT in patients suffering from delayed puberty compared with healthy girls supports that these genotypes delay pubertal onset in girls.

FSHR -29AA was found to be more prevalent among young women suffering from idiopathic premature ovarian insufficiency compared with healthy controls. None of the girls in the present study carried the combination of FSHR -29AA and FSHB -211TT. Thus, larger studies of adolescents and adult women are needed to clarify if this combination of genotypes causes markedly delayed puberty or even premature ovarian insufficiency.

The SNP in exon 10 of the gene encoding the FSH receptor (FSHR 2039A>G) did not affect pubertal onset either when evaluated separately or in combination with other polymorphisms. We did not include other polymorphisms in the FSH receptor or its promoter due to very low minor allele frequencies or high degree of linkage disequilibrium with FSHR 2039A>G.
Metabolites from adipose tissue.

Much focus has therefore been controlled by multiple pathways; e.g. kisspeptin, neurokinin B, and its connecting neurones drives the onset of puberty through the timing. BMI is a crude estimate of fat mass, and the effect of BMI in the present study. Thus, our findings indicate that even after adjusting for BMI)36. Part of the racial difference in age at pubertal onset indicates that the present study population is represented by healthy Chinese (CHB, minor allele frequency 51%) and Japanese (JPT, 47%), and less prevalent among Sub-Saharan Africans (YRI, 24%) compared with Western Europeans (CEU, 30%) (http://hapmap.ncbi.nlm.nih.gov).

Upstream of the pituitary gland, reactivation of the hypothalamus and its connecting neurones drives the onset of puberty through the hypothalamic-pituitary-gonadal (HPG) axis. Hypothalamic GnRH is controlled by multiple pathways; e.g. kisspeptin, neurokinin B, and metabolites from adipose tissue43,45. Much focus has therefore been placed upon childhood growth and adiposity in relation to pubertal timing. BMI is a crude estimate of fat mass, and the effect of FSHR -29G>A and FSHB -211G>T was not driven by differences in BMI in the present study. Thus, our findings indicate that even after central reactivation of the hypothalamus, down-stream processes in the HPG axis influence the peripheral attainment of secondary sexual characteristics.

Due to racial differences concerning age at pubertal onset and distribution of genotypes35,36, we have only included Caucasian girls in the final data analyses. The distribution of genotypes and the age at pubertal onset indicate that the present study population is representative for healthy Caucasian girls36,38. The HapMap project indicates that FSHR -29A is more prevalent among Chinese (CHB, minor allele frequency 51%) and Japanese (JPT, 47%), and less prevalent among Sub-Saharan Africans (YRI, 24%) compared with Western European (CEU, 30%) (http://hapmap.ncbi.nlm.nih.gov). The distribution fits well with the most recent longitudinal puberty study from the USA. Asian girls entered puberty later and black girls entered puberty at a younger age than white girls (remained after adjusting for BMI)36. Part of the racial difference in age at pubertal onset may be explained by different distributions of genotypes affecting FSH action.

In caucasian girls, pubertal onset has been reduced approximately one year during the past two decades35,36. This strong secular trend cannot be driven by genetic changes alone but is more likely caused by epigenetic changes, lifestyle factors, and/or environmental factors. Our finding of a strong regulatory effect of FSHR -29G>A on age at pubertal onset suggests that the pituitary-gonadal axis is a target for these factors responsible for the observed secular trend.

In this thoroughly characterized population of healthy Danish girls, pubertal onset was evaluated by the presence of palpable breast tissue, which is a current golden standard. This is in contrast to the majority of previous studies based on recollection of age at menarche. Our selective approach of assessing the effect of specific genes affecting FSH-signaling (a biological pathway essential for pubertal onset) minimises the risk of false positive associations. We have not assessed the effect of other polymorphisms.

In conclusion, we demonstrate here for the first time that age at pubertal onset in healthy girls is highly affected by genetic variation in promoters affecting FSH action. Breast development occurred 7.4 months later in healthy girls with the FSHR -29AA genotype compared with carriers of FSHR -29G+GA. This is the strongest genetic effect on age at pubertal onset in girls published to date.

**Methods**

**Participants.** Healthy girls. Participants were recruited as part of two population-based cohort studies: 1) The Copenhagen Puberty Study, detailed information about the study has been described previously15,36; a cross-sectional study conducted at ten schools in the Copenhagen area, 2006–2008. All pupils were invited (3102 girls) of whom 33% chose to participate, i.e. 1097 girls aged 6–20 years. 2) The Copenhagen Mother-Child Cohort, detailed information about the study has been described previously44,45, a longitudinal birth cohort following healthy Danish children. The children were examined during infancy, at mid-childhood and again yearly (one to three times) in 2010–2012 (mean age 11.0 years, range 6.0–15.0). In the present study-population, all girls (age 8–13 years) from the Copenhagen Mother-Child Cohort (n=548) and the cross sectional part of the Copenhagen Puberty Study (n=564) were included. No girls from our previously published study of FSH polymorphisms in pubertal girls (longitudinal part of the Copenhagen Puberty Study) were included43. Of the 1112 girls, a total of 148 were excluded from the present dataset because no DNA was available (n=94), one or both parents originated from a non-European country (n=46), intrauterine or endocrine disease (n=3), or due to lack of pubertal staging (n=5). The remaining 964 girls had no history of gynecological disorders.

**Patients with delayed puberty.** All girls registered with a diagnosis of delayed puberty (ICD-10: DE 30.0) at the Department of Growth and Reproduction, Rigshospitalet, Denmark were evaluated (n=168). DNA was available in 41 girls. Girls were excluded due to pubertal onset before age 13 years (n=14) or due to known cause of delayed puberty (e.g. Turner Syndrome and anorexia nervosa) (n=9). The remaining 18 girls with idiopathic delayed puberty were included in the study.

**Clinical examination.** Clinical evaluations were done by trained physicians and included pubertal staging of breast development according to Tanner’s classification evaluated by palpation46. Breast stage 2 or more was considered to be a marker of pubertal onset. Height was measured to the nearest 0.1 cm by a wall-mounted stadiometer (Holttain Ltd., Crymych, United Kingdom) and weight was measured to the nearest 0.1 kg using calibrated digital electronic scales (Sca Delta, Germany and Bisco model PERS 200, Denmark). Anthropometrics were available from 963 girls.

**Genotyping.** Peripheral blood (0.2 ml EDTA-preserved) was used for isolation of genomic DNA using the QuickGene-810 Nucleic Acid Isolation System (Fujiﬁlm, Life Science Products, Tokyo, Japan) and quantiﬁed on a NanoDrop ND-1000 spectrophotometer (Saveen Werner, Limhamn, Sweden).

All SNPs were analysed at LGC Genomics (LGC Genomics, Hoddesdon, UK) using their KASPar™ SNP genotyping assays, which facilitates bi-allelic discrimination through a competitive PCR and incorporation of a fluorescent resonance energy transfer quencher cassette. KASP™ genotyping assays were designed by LGC Genomics towards the following sequences: FSHB -211G>T (rs10835638), TATCAAAATTATAATTG[T]TTACAAAAATCTCAT; FSHR -29G>A (rs1394205), TCTTCTGAAATGTC[A/G]AAGAAATCCAGTTG; FSHR 2039A>G (rs6166), ATGTAATGGTGAACCA[C/T]TGGTGACTCTGGGA.

**Figure 2** | Distribution of genotypes FSHB -211G>T (A) and FSHR -29G>A (B) in Caucasian females (data from 1000 Genomes: www.1000genomes.org), as well as healthy Danish girls and 18 patients with delayed puberty from the present study population.
Discordance between the two methods were present in 6% (FSHR -29GG>T) and 12% (FSHR 2039A>G), respectively. In most cases re-inspection of gels showed ambiguous banding pattern in the RFLP analysis. A few remaining discordant cases were re-analysed, confirming all results in accordance with the KASP® genotyping.

Statistical analyses. To estimate the mean age (95% CI) at pubertal onset, we used probit analyses taking left, right – and interval censored data into account (SAS: proc lifereg). Probit analysis is a type of regression used with binomial response variables (in this study: breast development, yes/no)5. Based on cross sectional data, the probit analysis provides estimates of proportions of girls having entered puberty at different ages. In this study, longitudinal data of girls entering puberty during follow up is included in the probit analyses (interval censored data). In this way, all available information concerning age at pubertal onset is included. If a given girl entered puberty between two examinations during follow-up, we registered the last prepubertal examination (Tanner Stage B1) and the first post-pubertal examination (TS ≥ B2). The time between these examinations contains the true age at onset (interval-censored data, n = 95). If a girl had not entered puberty at her last examination (or only examination if she was only seen once), the current age at examination was known to be a lower bound for the true age at pubertal onset (right censored data, n = 245). If a girl had entered puberty at her first examination, the age at examination was known to be an upper bound for the true age at onset (left censored data, n = 624). We then included the genotypes in the model to estimate the related differences in mean age at pubertal onset (95% CI).

Two genetic models were used to assess the effect of genotypes on age at pubertal onset. In the recessive model, strong effects were expected only in the minor allele homozygous. These were compared with pooled wild-type and heterozygous (e.g. FSHR -29GG+GT vs. TT). The additive model assumed a co-dominant effect of alleles in which the heterozygotes should exhibit intermediate effect; regressions were calculated over, for example, FSHR -29GG vs. GA vs. AA (included as a continuous variable).

Based on initial analyses, we evaluated the combined effect of FSHR -29G>A and FSHB -211G>T. We tested if there was an additive effect of the minor alleles by including the subgroups as a continuous variable.

To estimate how much of the overall variance in age at pubertal onset was explained by the SNPs, we compared the results from two models. In the first model, we estimated the variance in the age at pubertal onset in a probit analysis in which genotypes were included as an explanatory variable. Secondly, we estimated the variance in a probit analysis in which genotypes was not included as an explanatory variable. The difference between the two variances is due to the SNPs included in the model.

To assess if the effect of the genotypes was driven by differences in BMI, we compared BMI levels between genotype sub-groups by independent samples t-test (2 groups) and One-way ANOVA (3 groups), and we adjusted the probit analysis for BMI by including this as a continuous variable.

To assess if the prevalence of susceptible genotypes was higher in patients with delayed puberty vs. healthy girls the x2 test was used. P ≤ 0.05 was considered statistically significant. Data were analysed separately by two investigators (CPF and JHP).

Ethical considerations. The COPENHAGEN Puberty Study (ClinicalTrials.gov ID: NCT01901977). The Copenhagen Mother-Child Cohort, and the genetic study of girls with delayed puberty were carried out in accordance with the protocols approved by the scientific ethical committee at The Capital Region of Denmark (KF 01 282214; V200.1996/90, KF 01 030/97/KF 01276357/H-1-2009-074, and KF1328087, respectively) as well as the Danish Data Protection Agency (2010-41-5042, 1997-58-01, 2012). In most cases re-inspection of gels showed ambiguous banding pattern in the RFLP analysis. A few remaining discordant cases were re-analysed, confirming all results in accordance with the KASP® genotyping.

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**Author contributions**
A.J., K.M.M., L.A., K.S., E.R.M., K.A., C.P.H. were responsible for study design. C.P.H., K.S., L.A., A.M., M.G.M., J.T., C.W.V., K.A. collected data. C.P.H., C.W.V., J.H.P., E.R.M., K.A., A.J. contributed to data analysis. All authors contributed to data interpretation and preparation of the manuscript.

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