



OPEN

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

SUBJECT AREAS:
GENETICS RESEARCH
PAEDIATRIC RESEARCH

Received
30 June 2014

Accepted
28 August 2014

Published
18 September 2014

Correspondence and requests for materials should be addressed to C.P.H. (casperhagen@gmail.com)

Casper P. Hagen, Kaspar Sørensen, Lise Aksglaede, Annette Mouritsen, Mikkel G. Mieritz, Jeanette Tinggaard, Christine Wohlfart-Veje, Jørgen Holm Petersen, Katharina M. Main, Ewa Rajpert-De Meyts, Kristian Almstrup & Anders Juul

Department of Growth and Reproduction, Rigshospitalet, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark.

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+GA, $p=0.003$. 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 differences¹. 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^{2,3}. Although genetic factors seem responsible for more than half of the phenotypic variation, knowledge of specific regulators of pubertal onset is limited⁴. Abnormal pubertal timing can be caused by rare mutations: e.g. *MKRN3*, *ESR1*, *KISS1*, *KISS1R*^{5–8} whereas common genetic variations in candidate loci (*KISS1*, *KISS1R*, *LIN28B*, *LIN28A*) do not seem to be involved in idiopathic central precocious puberty⁹. Additionally, large genome-wide association (GWA) studies have drawn attention to single nucleotide polymorphisms (SNP) affecting pubertal timing^{10–13}. Nevertheless, only approximately 15% of the variance in age at menarche can be explained by genetic variations identified in GWA studies¹⁴.

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 development¹⁵. 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 girls¹⁶.

Follicle-stimulating hormone (FSH) is essential for female reproductive maturation. FSH induces ovarian follicle growth and aromatase activity, hence, estradiol synthesis responsible for breast development¹⁷, and genetic variants in *FSHB* and *FSHR* have been reported as modulators of FSH action¹⁸.

FSHB -211G>T is located in the promoter of the gene encoding the FSH beta subunit. Reduced FSH production has been suggested in carriers of *FSHB* -211TT vs. GG^{19,20} due to altered affinity of the LHX3 transcription factor binding element²¹.

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)²² and they need more exogenous FSH for sufficient ovarian stimulation than *FSHR* 2039AA homozygotes^{23,24}.

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. GG²⁵. 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^{25,26}.

Recently, we observed that *FSHB* -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 study²⁷. 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: *FSHB* -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 *FSHB* -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 *FSHB* -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 menarche¹⁴. 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			Recessive model *	Additive model α
	GG	GT	TT	GG+GT vs. TT	GG vs. GT vs. TT
<i>FSHB</i> -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/m ²)	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)	$p = 0.246$	$p = 0.437$
<i>FSHR</i> -29G>A					
n	GG 512	GA 368	AA 74	GG+GA vs. AA	GG vs. GA vs. AA
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/m ²)	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)	$p = 0.003$	$p = 0.031$
<i>FSHR</i> 2039A>G					
n	AA 242	AG 504	GG 218	AA+AG vs. GG	AA vs. AG vs. GG
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/m ²)	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 wild-type 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).

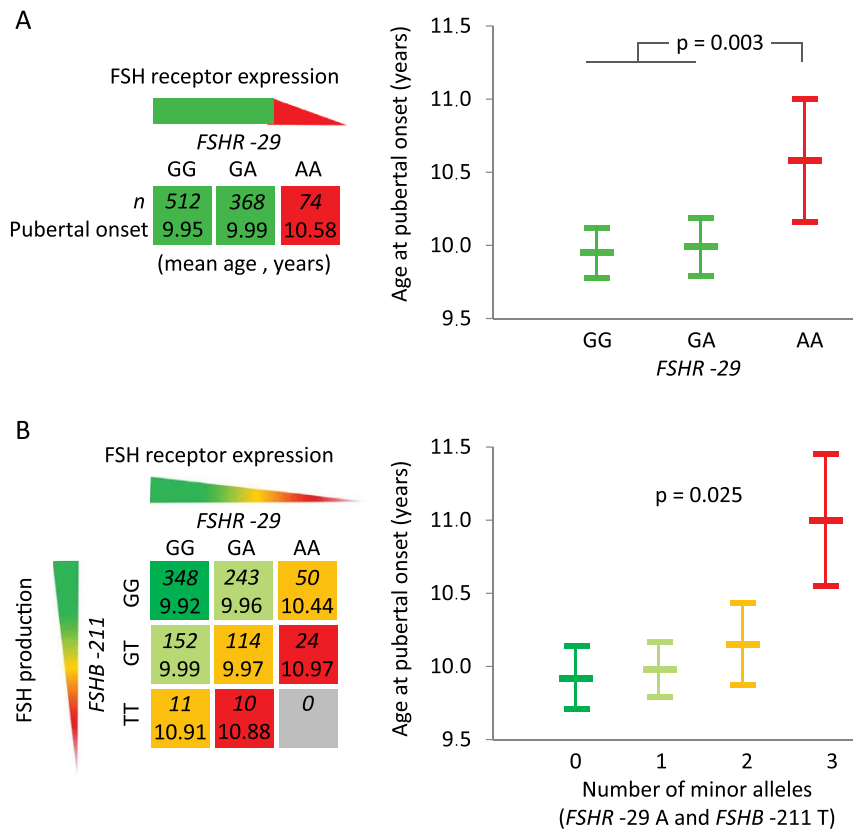


Figure 1 | Influence of *FSHR* -29G>A and *FSHB* -211G>T on age at pubertal onset in 964 healthy Danish girls. Left panel: The number of girls and their mean age at pubertal onset according to *FSHR* -29G>A genotypes (A) and the combined genotypes of *FSHR* -29G>A and *FSHB* -211G>T (B). In the combined model, we tested if there was an additive effect of the minor alleles across the genotypes (*FSHR* -29A and *FSHB* -211T). The fading green/yellow/red bars indicate intermediate FSH production and FSH receptor expression, respectively, in the heterozygotes compared with the wild-types and the minor allele homozygotes. The colours correspond to the subgroups of combined genotypes: 0 minor alleles: dark green; 1 minor allele: light green; 2 minor alleles: orange; 3 minor alleles: red; No girls had 4 minor alleles (C). Right panel: Mean age at pubertal onset (95% CI) according to *FSHR* -29G>A genotypes (A) and the combined genotypes of *FSHR* -29G>A and *FSHB* -211G>T (B). The combined model revealed additive effect of the minor alleles across the genotypes (B). The outline of the figure is inspired by Tuttelmann et al.⁴⁸.

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*¹⁰. 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 maturation²⁸, 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^{29–32}. 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 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 lines³³. 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^{25,26}. As in our study, intermediate effect was found in heterozygotes (*FSHR* -29AG)²⁵.

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 controls³⁴. 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 frequencies³³ or high degree of linkage disequilibrium with *FSHR* 2039A>G²⁴.



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.

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 tissue^{8,35}. 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 genotypes^{33,36}, 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 girls^{36–38}. 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 decades^{15,36}. 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 variation in 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* -29GG+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 of healthy Danish children and adolescents; 1) The COPENHAGEN Puberty Study, detailed information about the study has been described previously^{15,39}; a cross-sectional study conducted at ten schools in the Copenhagen area, 2006–2008. All pupils were invited (3102 girls) of whom 35% 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 previously^{40–42}; 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 peripubertal girls (longitudinal part of the COPENHAGEN Puberty Study) were included²⁷. 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), intracerebral 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 palpation⁴³. 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 (Holtain Ltd., Crymych, United Kingdom) and weight was measured to the nearest 0.1 kg using calibrated digital electronic scales (Seca 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 KASP™ 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), TATCAAATTTAATTT[G/T]TACAAAATCATCAT; *FSHR* -29G>A (rs1394205), TCTCTGCAAATGCAG[A/G]AAGAAATCAGGTGG; *FSHR* 2039A>G (rs61166), ATGTAAGTGGAAACCA[C/T]TGGTGACTCTGGGA. *FSHR* 2039A>G was genotyped in all 964 girls. In a few cases genotyping was impossible due to poor DNA quality, i.e. *FSHR* -29G>A and *FSHB* -211G>T were genotyped in 954/964 and 962/964 girls, respectively.

A subset of samples (COPENHAGEN Puberty Study, n=498) were re-genotyped for *FSHB* -211G>T and *FSHR* 2039A>G using restriction fragment length polymorphism (RFLP) analysis, according to previously described methods^{44–46}. In brief, DNA served as PCR template. Primers: *FSHB* -211G>T (GGAGCCAGATCATGAAATGTT and CGAAGTCCGATCGTAACCAAG) and *FSHR* 2039A>G (TTTGTGGTTCATCTGTGGCTGC and CAAAGGCAAGGACTGAATTATCATT). Fragments digested with *TatI* (Thermo Scientific, Erembodegem, Belgium) or *BsrI*



(New England Biolabs, Herts, UK). Genotypes were called by banding patterns. Samples with known genotypes were used as positive controls.

Discordance between the two methods were present in 6% (*FSHB*-211G>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)⁴⁷. 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 pre-pubertal 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 homozygotes. These were compared with pooled wild-type and heterozygotes (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 χ^2 test was used.

$P \leq 0.05$ was considered statistically significant. Data were analysed separately by two investigators (CPH and JHP).

Ethical considerations. The COPENHAGEN Puberty Study (ClinicalTrials.gov ID: NCT01411527), 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, 1997-1200-074/2005-41-5545/2010-41-4757, 2006-41-7251, respectively). The study was conducted in accordance with the second Helsinki Declaration. All children and parents received written information, and informed consent was obtained from all participants.

- Parent, A. S. *et al.* The timing of normal puberty and the age limits of sexual precocity: variations around the world, secular trends, and changes after migration. *Endocr. Rev.* **24**, 668–693 (2003).
- Carel, J. C. & Leger, J. Clinical practice. Precocious puberty. *N. Engl. J. Med.* **358**, 2366–2377 (2008).
- Golub, M. S. *et al.* Public health implications of altered puberty timing. *Pediatrics* **121** Suppl 3, S218–S230 (2008).
- Sorensen, K. *et al.* Birth size and age at menarche: a twin perspective. *Hum. Reprod.* **28**, 2865–2871 (2013).
- Abreu, A. P. *et al.* Central precocious puberty caused by mutations in the imprinted gene *MKRN3*. *N. Engl. J. Med.* **368**, 2467–2475 (2013).
- Quaynor, S. D. *et al.* Delayed puberty and estrogen resistance in a woman with estrogen receptor alpha variant. *N. Engl. J. Med.* **369**, 164–171 (2013).
- Teles, M. G. *et al.* A GPR54-activating mutation in a patient with central precocious puberty. *N. Engl. J. Med.* **358**, 709–715 (2008).
- Topaloglu, A. K. *et al.* Inactivating *KISS1* mutation and hypogonadotropic hypogonadism. *N. Engl. J. Med.* **366**, 629–635 (2012).
- Tommiska, J. *et al.* *LIN28B*, *LIN28A*, *KISS1*, and *KISS1R* in idiopathic central precocious puberty. *BMC. Res. Notes* **4**, 363 (2011).

- Ong, K. K. *et al.* Genetic variation in *LIN28B* is associated with the timing of puberty. *Nat. Genet.* **41**, 729–733 (2009).
- Perry, J. R. *et al.* Meta-analysis of genome-wide association data identifies two loci influencing age at menarche. *Nat. Genet.* **41**, 648–650 (2009).
- Sulem, P. *et al.* Genome-wide association study identifies sequence variants on 6q21 associated with age at menarche. *Nat. Genet.* **41**, 734–738 (2009).
- Elks, C. E. *et al.* Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. *Nat. Genet.* **42**, 1077–1085 (2010).
- Perry, J. R. Parent-of-origin-specific allelic associations among 106 genomic loci for age at menarche. *Nature* doi:10.1038/nature13545 (2014).
- Akslae, L., Sorensen, K., Petersen, J. H., Skakkebaek, N. E. & Juul, A. Recent decline in age at breast development: the Copenhagen Puberty Study. *Pediatrics* **123**, e932–e939 (2009).
- Tanner, J. M. *Growth at adolescence* (Blackwell & Mott Ltd, Oxford, 1962).
- Jenner, M. R., Kelch, R. P., Kaplan, S. L. & Grumbach, M. M. Hormonal changes in puberty. IV. Plasma estradiol, LH, and FSH in prepubertal children, pubertal females, and in precocious puberty, premature thelarche, hypogonadism, and in a child with a feminizing ovarian tumor. *J. Clin. Endocrinol. Metab* **34**, 521–530 (1972).
- Simoni, M. & Casarini, L. Mechanisms in endocrinology: Genetics of FSH action: a 2014-and-beyond view. *Eur. J. Endocrinol.* **170**, R91–107 (2014).
- La, M. A. *et al.* The combination of genetic variants of the *FSHB* and *FSHR* genes affects serum FSH in women of reproductive age. *Hum. Reprod.* **28**, 1369–1374 (2013).
- Hoogendoorn, B. *et al.* Functional analysis of human promoter polymorphisms. *Hum. Mol. Genet.* **12**, 2249–2254 (2003).
- Benson, C. A., Kurz, T. L. & Thackray, V. G. A Human *FSHB* Promoter SNP Associated with Low FSH Levels in Men Impairs LHX3 Binding and Basal *FSHB* Transcription. *Endocrinology* (2013).
- Casarini, L. *et al.* *FSHR* polymorphism p.N680S mediates different responses to FSH in vitro. *Mol. Cell Endocrinol.* **393**, 83–91 (2014).
- Nordhoff, V. *et al.* Effects of the FSH receptor gene polymorphism p.N680S on cAMP and steroid production in cultured primary human granulosa cells. *Reprod. Biomed. Online.* **23**, 196–203 (2011).
- Perez, M. M. *et al.* Ovarian response to follicle-stimulating hormone (FSH) stimulation depends on the FSH receptor genotype. *J. Clin. Endocrinol. Metab* **85**, 3365–3369 (2000).
- Desai, S. S. *et al.* Follicle-stimulating hormone receptor polymorphism (G-29A) is associated with altered level of receptor expression in Granulosa cells. *J. Clin. Endocrinol. Metab* **96**, 2805–2812 (2011).
- Nakayama, T. *et al.* Mutation of the follicle-stimulating hormone receptor gene 5'-untranslated region associated with female hypertension. *Hypertension* **48**, 512–518 (2006).
- Hagen, C. P. *et al.* *FSHB*-211 and *FSHR* 2039 are associated with serum levels of follicle-stimulating hormone and antimullerian hormone in healthy girls: a longitudinal cohort study. *Fertil. Steril.* **100**, 1089–1095 (2013).
- Rosenfield, R. L., Bordini, B. & Yu, C. Comparison of detection of normal puberty in girls by a hormonal sleep test and a gonadotropin-releasing hormone agonist test. *J. Clin. Endocrinol. Metab* **98**, 1591–1601 (2013).
- Kumar, T. R., Wang, Y., Lu, N. & Matzuk, M. M. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* **15**, 201–204 (1997).
- Dierich, A. *et al.* Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13612–13617 (1998).
- Matthews, C. H. *et al.* Primary amenorrhoea and infertility due to a mutation in the beta-subunit of follicle-stimulating hormone. *Nat. Genet.* **5**, 83–86 (1993).
- Aittomaki, K. *et al.* Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* **82**, 959–968 (1995).
- Wunsch, A. *et al.* Single-nucleotide polymorphisms in the promoter region influence the expression of the human follicle-stimulating hormone receptor. *Fertil. Steril.* **84**, 446–453 (2005).
- Achrekar, S. K., Modi, D. N., Meherji, P. K., Patel, Z. M. & Mahale, S. D. Follicle stimulating hormone receptor gene variants in women with primary and secondary amenorrhea. *J. Assist. Reprod. Genet.* **27**, 317–326 (2010).
- Welt, C. K. *et al.* Recombinant human leptin in women with hypothalamic amenorrhea. *N. Engl. J. Med.* **351**, 987–997 (2004).
- Biro, F. M. *et al.* Onset of Breast Development in a Longitudinal Cohort. *Pediatrics* (2013).
- Herman-Giddens, M. E. *et al.* Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. *Pediatrics* **99**, 505–512 (1997).
- Sun, S. S. *et al.* National estimates of the timing of sexual maturation and racial differences among US children. *Pediatrics* **110**, 911–919 (2002).
- Sorensen, K., Akslae, L., Petersen, J. H. & Juul, A. Recent changes in pubertal timing in healthy Danish boys: associations with body mass index. *J. Clin. Endocrinol. Metab* **95**, 263–270 (2010).
- Chellakootty, M. *et al.* Inhibin A, Inhibin B, Follicle-Stimulating Hormone, Luteinizing Hormone, Estradiol, and Sex Hormone-Binding Globulin Levels in 473 Healthy Infant Girls. *Journal of Clinical Endocrinology Metabolism* **88**, 3515–3520 (2003).



41. Boisen, K. A. *et al.* Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries. *Lancet* **363**, 1264–1269 (2004).
42. Wohlfahrt-Veje, C. *et al.* Body fat throughout childhood in 2647 healthy Danish children: agreement of BMI, waist circumference, skinfolds with dual X-ray absorptiometry. *Eur. J. Clin. Nutr.* **68**, 664–670 (2014).
43. Marshall, W. A. & Tanner, J. M. Variations in pattern of pubertal changes in girls. *Arch. Dis. Child* **44**, 291–303 (1969).
44. Grigорова, M., Punab, M., Ausmees, K. & Laan, M. FSHB promoter polymorphism within evolutionary conserved element is associated with serum FSH level in men. *Hum. Reprod.* **23**, 2160–2166 (2008).
45. Sudo, S. *et al.* Genetic and functional analyses of polymorphisms in the human FSH receptor gene. *Mol. Hum. Reprod.* **8**, 893–899 (2002).
46. Pengo, M. *et al.* FSH receptor gene polymorphisms in fertile and infertile Italian men. *Reprod. Biomed. Online* **13**, 795–800 (2006).
47. FINNEY, D. J. & STEVENS, W. L. A table for the calculation of working probits and weights in probit analysis. *Biometrika* **35**, 191–201 (1948).
48. Tuttelmann, F. *et al.* Combined effects of the variants FSHB -211G>T and FSHR 2039A>G on male reproductive parameters. *J. Clin. Endocrinol. Metab* **97**, 3639–3647 (2012).

Acknowledgments

The authors are grateful to the participating children and all colleagues who were involved in the COPENHAGEN Puberty Study and The Copenhagen Mother-Child Cohort. Special thanks to H. Kelkeland, M. Dalgaard, B. Hansen, H. Hansen, I. Garn, and the staff of the hormone laboratory for their expert technical assistance. The study received financial

support from the Danish Council for Independent Research (DFF-1331-00113), Danish Agency for Science, Technology and Innovation (09-067180), The Capital Region of Denmark's Research Fund for Health Research (R129-A3966), and The Kirsten and Freddy Johansen Foundation.

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.

Additional information

Disclosure statement: The authors have nothing to disclose.

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hagen, C.P. *et al.* Pubertal Onset in Girls is Strongly Influenced by Genetic Variation Affecting FSH Action. *Sci. Rep.* **4**, 6412; DOI:10.1038/srep06412 (2014).



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/4.0/>