Human Reproduction, Vol.31, No.4 pp. 897–904, 2016
Advanced Access publication on February 23, 2016 doi:10.1093/humrep/dew022

human reproduction

ORIGINAL ARTICLE ***Reproductive genetics***

Genetic variations altering FSH action affect circulating hormone levels as well as follicle growth in healthy peripubertal girls

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Submitted on September 7, 2015; resubmitted on December 15, 2015; accepted on January 22, 2016

STUDY QUESTION: Do variants of the genes encoding follicle stimulating hormone (FSH) beta subunit (B) and FSH receptor (R) impact circulating reproductive hormone levels and ovarian follicle maturation in healthy peripubertal girls?

SUMMARY ANSWER: *FSHB* and *FSHR* genetic variants exert, alone or their combination, distinct effects on reproductive hormone levels as well as ovarian follicle maturation in healthy peripubertal girls.

WHAT IS KNOWN ALREADY: *FSHB* and *FSHR* genetic variants impact reproductive hormone levels as well as associated pathologies in women. While *FSHR* c. 2039A>G is known to alter gonadotrophin levels in women, *FSHR* c.-29G>A has not yet been shown to exert effect and there are conflicting results concerning *FSHB* c.-211G>T.

STUDY DESIGN, SIZE, DURATION: This population-based study included 633 girls recruited as part of two cohorts, the COPENHAGEN Puberty Study (2006–2014, a cross-sectional and ongoing longitudinal study) and the Copenhagen Mother–Child Cohort (1997–2002, including transabdominal ultrasound (TAUS) of the ovaries in a subset of 91 peripubertal girls).

PARTICIPANTS/MATERIALS, SETTING, METHODS: Clinical examinations, including pubertal breast stage (Tanner's classification B1–B5) were performed. Circulating levels of FSH, luteinizing hormone (LH), estradiol, anti-Mullerian hormone (AMH) and inhibin-B were assessed by immunoassays. In a subset of the girls ($n = 91$), ovarian volume and the number/size of antral follicles were assessed by TAUS. Genotypes were determined by competitive PCR.

MAIN RESULTS AND THE ROLE OF CHANCE: *FSHR* c.2039A>G minor alleles were positively associated with serum FSH ($\beta = 0.08$, $P = 0.004$), LH ($\beta = 0.06$, $P = 0.012$) and estradiol ($\beta = 0.06$, $P = 0.017$) (adjusted for Tanner stages). In a combined model, *FSHR* c.-29G>A and *FSHR* c.2039A>G alleles were positively associated with FSH levels in early-pubertal girls (B2 + B3, $n = 327$, $r = 0.1$, $P = 0.02$) and in young adolescents (B4 + B5, $n = 149$, $r = 0.2$, $P = 0.01$). Serum AMH and inhibin B levels were not significantly influenced by the single nucleotide polymorphisms (SNPs). Single SNPs were not associated with follicles counts, however, a cumulative minor allele count (*FSHB* c.-211 G>T and *FSHR* c.-29G>A) was negatively associated with the number of large follicles (≥5 mm) ($n = 91$, $P = 0.04$) (adjusted for Tanner stages).

LIMITATIONS, REASONS FOR CAUTION: Since we studied girls and young adolescents during pubertal transition, our study may not be fully comparable with previous studies on *FSHB* and *FSHR* variants in adult women. The group of young adolescents (Tanner B4 + B5) reflects the endocrine situation in adult women best, however, the group is not large enough to contribute substantially to the conflicting results concerning the influence of *FSHB* c.-211G>T in adult women. Furthermore, we have no information about the exact day of the menstrual cycle in the subgroup of girls with menarche.

WIDER IMPLICATIONS OF THE FINDINGS: The sex-specific interaction of *FSHB* and *FSHR* genetic variants and physiological as well as pathological conditions is being increasingly elucidated. The variant triplet set might serve as diagnostic and pharmacogenetic marker. For the first time, we show an additional effect of *FSHR* c.-29G>A on serum FSH levels in healthy girls. Moreover, morphological data suggest impaired

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FSH-induced maturation of ovarian follicles in minor allele carriers of *FSHB* c.-211G>T and *FSHR* c.-29G>A. This may explain previous findings of delayed pubertal onset in these girls.

STUDY FUNDING/COMPETING INTEREST(S): Funding was provided by the Danish Agency for Science, Technology and Innovation (09-067180), Danish Ministry of the Environment, CeHoS (MST-621-00065), Capital Region of Denmark (December 2011), Ministry of Higher Education and Science (DFF—1331-00113) and EDMaRC (Danish Ministry of Health). A.S.B. was funded from December 2015 by ReproUnion (EU Interreg Öresund-Kattegat-Skagerrak). The authors declare no conflict of interest.

Key words: FSH / FSH receptor / single nucleotide polymorphism / puberty / ovarian follicle

Introduction

Follicle-stimulating hormone (FSH) is a key player in human reproduction, promotes granulosa cell proliferation and differentiation as well as estradiol synthesis (Macklon and Fauser, 1998). Ovarian follicle maturation, including initial recruitment from the primordial follicle pool and cyclic follicle recruitment, development and selection, requires concerted actions of inhibiting and activating factors (Kerr *et al.*, 2013). From fetal life and through the reproductive lifespan, recruitment of primordial follicles represents a continuous FSH-independent process while subsequent follicle growth is FSH-sensitive (McGee and Hsueh, 2000). Patients with inactivating mutations of the FSH beta subunit (*FSHB*) and the FSH receptor (*FSHR*) genes develop follicles up to pre-antral stages, but further maturation is disrupted (Matthews *et al.*, 1993; Aittomaki *et al.*, 1995; Kumar *et al.*, 1997; Dierich *et al.*, 1998). As a consequence, they suffer from premature ovarian insufficiency causing absent pubertal development and primary amenorrhoea (Huhtaniemi and Themmen, 2005). Although extensive estradiol production is limited to larger antral follicles surrounded by multiple layers of granulosa cells (>8 mm diameter) (Andersen *et al.*, 2010), FSH-receptors along with pivotal steroidogenic enzymes (17βHSD1 and CYP19A1) are present even in small antral follicles. This suggests FSH responsiveness and the capability to aromatize androgens to estrogens even in small antral follicles (Oktay *et al.*, 1997; Kristensen *et al.*, 2015). The presence of small antral follicles in ovaries of girls prior to puberty has previously been observed by transabdominal ultrasound (TAUS) (Peters *et al.*, 1978; Stanhope *et al.*, 1985). Recently, magnetic resonance imaging and high resolution TAUS have even revealed the presence of medium/large antral follicles (5–9 mm) in all healthy prepubertal girls (Hagen et al., 2014, 2015). Though, substantial progress has been made during the last decades, follicular dynamics during childhood and young adolescence remains poorly understood.

Single nucleotide polymorphisms (SNPs) located within or in the close vicinity of the genes encoding FSHB and FSHR have been shown to affect FSH production (*FSHB* c.-211G>T, rs10835638) (Webster *et al.*, 1995; Hoogendoorn *et al.*, 2003) and sensitivity/expression of its receptor *in vitro* (*FSHR* c.2039A>G, rs6166 and *FSHR* c.-29G>A, rs1394205, respectively) (Wunsch *et al.*, 2005; Nakayama *et al.*, 2006; Desai *et al.*, 2013; Casarini *et al.*, 2014). In agreement with these findings, minor allele carrier status of the receptor SNPs was associated with lower ovarian response to ovarian stimulation (Desai *et al.*, 2011, 2013; Lledo *et al.*, 2013). Furthermore, *FSHR* c.2039A>G was associated with serum FSH concentrations in adult women (Greb *et al.*, 2005), whereas *FSHR* c.-29G>A did not seem to affect FSH levels (Wunsch *et al.*,2005; Achrekar *et al.*, 2009). The possible impact of *FSHB* c.-211G>T on FSH levels in adult women are conflicting and based on relatively small studies (La Marca *et al.*, 2013; Schüring *et al.*, 2013). However, recent genome-wide association studies (GWAS) underline the importance of the *FSHB* c.-211G>T variant and those in close vicinity on female gonadotrophin levels, reproductive health and ageing (Day *et al.*, 2015a,b; Hayes *et al.*, 2015).

Beside nutritional, environmental and socioeconomic factors, genetic factors seem responsible for more than half of the phenotypic variation of pubertal onset (Sørensen *et al.*, 2013). We have recently observed that *FSHB* c.-211G>T and *FSHR* c.-29G>A genotypes strongly affect the age at pubertal onset in girls (Hagen *et al.*, 2014, 2015). Furthermore, in a pilot study of healthy peripubertal girls, we observed that *FSHB* c.-211G>T and *FSHR* c. 2039A>G affect circulating levels of FSH and AMH prior to pubertal onset (Hagen *et al.*, 2013).

We here report association of circulating levels of FSH, luteinizing hormone (LH), inhibin B, estradiol and AMH with *FSHB*/*FSHR* genotypes depending on pubertal development in 633 healthy girls. Furthermore, in a subset of the girls, we evaluated the association between their *FSHB* and *FSHR* genotypes and ovarian follicle morphology assessed by transabdominal ultrasound.

Materials and Methods

Healthy females

Participants were recruited as part of two population-based cohort studies of healthy Danish children and adolescents: (i) the COPENHAGEN Puberty Study and (ii) the Copenhagen Mother–Child Cohort. In subgroups of the girls, we have previously described reproductive hormone levels (Aksglaede *et al.*, 2009; Hagen *et al.*, 2012), genotypes (Hagen *et al.*, 2014) and ovarian morphology (Hagen *et al.*, 2015) separately. Only in a pilot study ($n = 78$), we have reported associations of *FSHR* c. 2039A>G and *FSHB* c.-211G>T with circulating FSH and AMH (Hagen *et al.*, 2013). In the present study, we have compiled all available data (633 girls) and for the first time evaluated whether *FSHR* c. 2039A>G, *FSHB* c.-211G>T and *FSHR* c.-29G>A are associated with reproductive hormone levels (FSH, LH, AMH, inhibin B, estradiol) as well as ovarian morphology in a large number of healthy peripubertal girls.

Detailed information about The COPENHAGEN Puberty Study has been described previously (Aksglaede *et al.*, 2009; Sorensen *et al.*, 2010; Hagen *et al.*, 2012). It was a combined cross-sectional and ongoing longitudinal study conducted at 10 schools in the Copenhagen area, 2006-014. All pupils (3102 girls) were invited and 35% chose to participate, i.e. 1097 girls aged 6–20 years. In the longitudinal part of the study, 108 girls and adolescents from 2 of the included schools were examined twice a year. In the present study, we included all girls (8–13 years) from the cross-sectional part ($n = 440$) (Hagen *et al.*, 2014) and girls from the longitudinal part of the COPENHAGEN Puberty study ($n = 75$) (Hagen *et al.*, 2013). A total of 145 girls were excluded because no DNA was available, or one or both

parents originated from a non-European country, or intracerebral or endocrine disease was present, or there was a lack of pubertal staging. To evaluate associations between genotypes and ovarian morphology, we included a nested cohort of The Copenhagen Mother–Child Cohort. Detailed information about this study has been described previously (Chellakooty *et al.*, 2003; Hagen *et al.*, 2015). Participants were Danish children born at three hospitals in the Copenhagen area 1997–2002. The children were examined at several time-points during infancy and childhood, and 1293 peripubertal children (584 girls) agreed to participate in an ongoing longitudinal study with annual examinations (participation rate 43%). The selection criterion for the nested cohort was high attendance rate at previous examinations (≥5 previous examinations). Of 129 invited girls, 121 consented and underwent an extended examination including TAUS of the ovaries. The number of girls available with combined data of genotypes and reproductive hormone levels as well as genotypes and the total number of ovarian follicles were 118 and 91, respectively. All girls were Caucasian Danes and no girls had a history of endocrine, gynecological or cerebral illness.

Clinical examination

Clinical evaluations were performed by trained physicians and included pubertal staging of breast development according to Tanner's classification (breast stage, B1–B5) evaluated by palpation (Marshall and Tanner, 1969). Breast stage 2 (B2, 'elevation of breast and papilla as a small mound, enlargement of areola diameter') was considered to be a marker of puberty.

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 using the KASP^{TM} SNP genotyping assays, which facilitate bi-allelic discrimination through competitive PCR and incorporation of a fluorescent resonance energy transfer quencher cassette, either at LGC Genomics (Hoddesdon, UK) or at the genetic laboratory at our department. KASP^{TM} genotyping assays were designed towards the following sequences: *FSHB* c.-211G>T (rs10835638), TATCAAATTTAATTT[G/T]TACAAAA TCATCAT; *FSHR* c.-29G>A (rs1394205), TCTCTGCAAATGCAG[A/G] AAGAAATCAGGTGG; *FSHR* c.2039A>G (rs6166), ATGTAAGTGGAA CCA[C/T]TGGTGACTCTGGGA. *FSHB* c.-211G>T, *FSHR* c.-29G>A, *FSHR* c.2039A>G were available from 633 girls of Caucasian ancestry.

Reproductive hormone assays

All blood samples were drawn between 8:00 a.m. and 2:00 p.m. from an antecubital vein, clotted and centrifuged; serum was stored at −20°C until hormone analyses. All samples were analysed in the same laboratory with blinding for age and pubertal stage.

Serum FSH and LH levels were measured by time-resolved immunofluorometric assays (Delfia; PerkinElmer, Boston, MA, USA) with detection limits of 0.06 and 0.05, respectively. Intra- and inter-assay coefficients of variation (CVs) were <5%. Serum AMH levels were determined using the Beckman Coulter enzyme immunometric assay generation I (IOT, Immunotech, Beckman Coulter Ltd., Marseilles, France) with a detection limit of 2.0 pmol/l. The intra-assay CVs were <10.8 and 9.2% at 18 and 99 pmol/l, respectively. Serum concentrations of inhibin B were measured using the Beckman Coulter GenII assay with a detection limit of 3 pg/ml, and inter-assay CV <11%. Serum estradiol was measured by radioimmunoassay (Pantex, Santa Monica, CA, USA), with a detection limit 18 pmol/l, and intra- and inter-assay CVs were <8 and 13%, respectively.

Ovarian morphology

Ovarian morphology was assessed by TAUS (Hagen *et al.*, 2015). In short, TAUS examinations were performed by a single experienced operator using a Voluson E8 Ultrasound System (GE Healthcare Medical Systems, Zipf, Austria) with a multi-frequency transabdominal probe (RM6C, 3–8 MHz). Analyses were performed concomitantly by two experienced operators.

Follicle numbers (≥1 mm) were evaluated by Tomographic Ultrasound Imaging where a 3D model of the ovary was sliced (4 mm thickness) and follicles were manually counted in subgroups (Deb *et al.*, 2010): small (1.0–4.4 mm), medium (4.5–9.4 mm) and large (≥9.5 mm) (3D TAUS). We report the sum of follicles from both ovaries ($n = 91$ girls).

Statistical analyses

Our study contains data from the longitudinal part of The COPENHAGEN Puberty study ($n = 75$). The raw dataset of each individual contains data collected in several examinations as puberty progressed. To evaluate the effect of SNPs on reproductive hormone levels, observations were grouped according to Tanner stage for each individual. If multiple observations within a specific Tanner subgroup were available, a mean value was calculated. This mean value was introduced into the model and used like the single-occasion observations of the cross-sectional part of the COPENHAGEN Puberty Study and the nested cohort of Copenhagen Mother–Child Cohort when performing statistical analyses.

Separate effect of SNPs

To evaluate the effect of SNPs independent of pubertal development, we performed multiple regression analyses including a SNP (*FSHB* c.-211G>T, *FSHR* c.-29G>A and *FSHR* c.2039A>G, respectively WW versus WM versus MM) and Tanner stages (B1–B5) as predictor variables. The unique contribution of each genetic variant, the effect size (β), was calculated. To evaluate whether the effect of the SNPs was present only in certain stages of puberty, we divided the girls into Tanner subgroups and performed simple linear regression analyses (SNP as predictor) for each Tanner subgroup. With regard to the physiological development and sufficient group sizes, we formed subgroups according to Tanner stages (B1/prepubertal, B2 + B3/early-pubertal, B4 + B5/young adolescents). In case of low minor allele frequency and failure to obtain normal distribution (LH for *FSHB* c.-211G>T), the effect was evaluated by the Mann–Whitney *U* test.

Combined effect of SNPs

To evaluate the combined effect of genetic variation altering FSH action on circulating serum FSH levels, we included only *FSHR* SNPs, expecting a compensatory increase in FSH levels in girls with an increasing number of minor alleles (due to reduced receptor sensitivity, *FSHR* c.-29A and *FSHR* c.2039G (Simoni and Casarini, 2014)). To evaluate the combined effect of genetic variations concerning the distribution of ovarian follicles, we included (i) *FSHB* c.-211G>T and *FSHR* c.-29G>A, previously shown to affect age at pubertal onset in girls (Hagen *et al.*, 2014), and (ii) all three SNPs, expecting reduced FSH action and hence attenuated follicle growth in girls with increasing numbers of minor alleles. In analyses of Tanner subgroups, normal variation was not obtained and Spearman's rho was used to evaluate whether genotypes were associated with follicle numbers.

In case of skewed distribution, we performed log-transformation of hormone and morphological variables to obtain a normal distribution. A *P*-value ≤0.05 was considered statistically significant.

Ethical considerations

The COPENHAGEN Puberty Study (ClinicalTrials.gov ID: NCT01411527) and The Copenhagen Mother–Child Cohort were carried out in accordance

with the protocols approved by the scientific ethical committee at The Capital Region of Denmark (KF 01 284; 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). All children and parents received written information, and informed consent was obtained from all participants.

Results

FSHB/FSHR genotypes and reproductive hormone levels

FSHR c.2039A>G minor alleles were positively associated with serum FSH ($\beta = 0.08$, $P = 0.004$), LH ($\beta = 0.06$, $P = 0.012$) and estradiol ($\beta = 0.06$, $P = 0.017$) (adjusted for Tanner stages, data not shown). When evaluating each Tanner subgroup separately, the *FSHR* c.2039A>G genotype was associated with circulating FSH levels in girls after pubertal onset [B2 + B3: $n = 327$, $r = 0.1$, $P = 0.02$ and B4 + B5: $n = 149$, $r = 0.2$, $P = 0.01$; Δ serum FSH (median, WW versus MM) +36% (B2 + B3) and +15% (B4 + B5); Table I and Fig. 1 upper part]. *FSHR* c.-29G>A was not associated with serum FSH levels when adjusted for Tanner stages and the effect of other SNPs ($\beta = 0.01$, $P = 0.64$, data not shown). However, in a combined model, *FSHR* c.-29G>A modulated the dominant effect of *FSHR* c.2039A>G resulting in a significant positive association with serum FSH levels in early-pubertal girls (B2 + B3, $n = 327$, $r = 0.1$, $P = 0.02$) and in young adolescents (B4 + B5, $n = 149$, $r = 0.2$, $P = 0.01$, Fig. 1). The effect size of the *FSHR* c.2039A>G in the latter subgroup was about four times larger than the effect of *FSHR* c.-29G>A.

FSHB c.-211G>T did not affect FSH levels significantly, however, the minor allele count was positively associated with serum LH ($\beta = 0.08$, $P = 0.0001$) (adjusted for Tanner stages, data not shown). The association was present in all Tanner subgroups: minor allele carriers had significantly increased LH levels (GG versus GT/TT; B1, $n = 221$, $P = 0.02$; B2 + B3, $n = 327$, $P = 0.04$; B4 + B5, $n = 149$, $P = 0.02$). Furthermore, the LH/FSH ratio was positively associated with *FSHB* c.-211G>T minor allele count ($P = 5 \times 10^{-6}$) (adjusted for Tanner stages, data not shown) again in all subgroups (B1, $n = 221$, $P = 0.006$; B2 + B3, $n = 327$, $P = 0.009$; B4 + 5, $n = 149$, $P = 0.01$).

AMH and inhibin B were not significantly influenced by the SNPs or their combination (data not shown).

FSHB/FSHR genotypes and ovarian follicle distribution

Single SNPs were not associated with follicle counts (data not shown), but we observed a negative association between the cumulative minor allele count (*FSHB* c.-211 G>T and *FSHR* c.-29G>A) and the number of large follicles (≥5 mm) ($n = 91$, $P = 0.04$) as well as the ratio of small to large follicles (1–4 versus ≥5 mm) ($n = 91$, $P = 0.04$) (adjusted for Tanner stages, data not shown). When evaluating each Tanner subgroup, the cumulative minor allele counts of the three variants *FSHB* c.-211 G>T, *FSHR* c.2039A>G and *FSHR* c.-29G>A (0–4 counts) were negatively associated with the number of large follicles (≥5 mm) [Spearman's rho (r) = −0.8, $P = 0.005$] (Supplementary data, Fig. S1A) and positively associated with the ratio of small to large follicles (1–4 versus ≥5 mm) [Spearman's rho (r) = 0.7, $P = 0.05$] in prepubertal girls (B1, $n = 11$ girls) (Supplementary data, Fig. S1B). Neither variant genotype nor their combinations were associated with follicle counts/ratio in other Tanner groups. The inherent distribution of alleles in the cumulative groups is shown in Supplementary data, Table SI and no obvious trends in the distribution of alleles across genotypes or their zygosity were observed.

Discussion

In the largest and most comprehensive study of *FSHB* and *FSHR* effects on ovarian function in healthy girls, we observed for the first time that variations in the genes encoding the FSH receptor and FSHβ subunit were associated with circulating FSH and estradiol levels (*FSHR* c.2039A>G) as well as LH levels (*FSHR* c.2039A>G and *FSHB* c.-211G>T). Moreover, *FSHR* c.2039A>G and *FSHR* c.-29G>A exerted a combined effect on circulating FSH levels in young adolescents.

Table I Serum FSH levels according to Tanner's breast stages and *FSHB*/ *FSHR* genotypes.[a]

					FSH [U/l]			Additive model	
		n (WW|WM|MM)[a]			WW	WM	MM	Minor allele effect	*P*-Value
B1	*FSHB* −211G>T	146	68	7	1.4 (0.0–5.3)	1.4 (0.4–8.0)	1.7 (0.4–2.5)	0.2[b]	0.23
	FSHR −29G>A	110	85	26	1.4 (0.3–6.6)	1.5 (0.1–8.0)	1.5 (0.0–4.9)	0.1[c]	0.55
	FSHR 2039A>G	56	111	54	1.4 (0.0–4.0)	1.4 (0.4–8.0)	1.4 (0.4–6.6)	0.1[c]	0.54
B2 + B3	*FSHB* −211G>T	226	97	4	3.0 (0.4–8.2)	3.5 (0.3–7.6)	3.5 (2.8–5.5)	0.1[b]	0.61
	FSHR −29G>A	178	123	26	3.1 (0.3–8.2)	3.5 (0.3–6.9)	3.4 (0.5–6.0)	0.0[c]	0.99
	FSHR 2039A>G	97	160	70	2.8 (0.4–7.2)	3.1 (0.5–7.7)	3.8 (0.3–8.2)	0.3[c]	0.02
B4 + B5	*FSHB* −211G>T	93	53	3	4.9 (0.6–9.5)	5.0 (1.4–11.0)	4.9 (3.3–5.1)	0.1[b]	0.80
	FSHR −29G>A	80	59	10	4.9 (0.6–9.5)	4.9 (1.4–11.0)	5.2 (1.4–8.4)	0.2[c]	0.51
	FSHR 2039A>G	43	68	38	4.6 (0.6–7.4)	4.9 (1.4–9.5)	5.3 (1.4–11.0)	0.5[c]	0.01

Data presented as median (range).
[a]W: wild-type allele; M, minor allele (*FSHB* −211 T, *FSHR* c.-29A, *FSHR* c.2039G); 75 girls from the longitudinal part of the COPENHAGEN Puberty Study contributed with one observation from each Tanner subgroup.
[b]Minor allele effect: Mann–Whitney *U* (WW versus WM + MM).
[c]Pearson's rho (*r*).

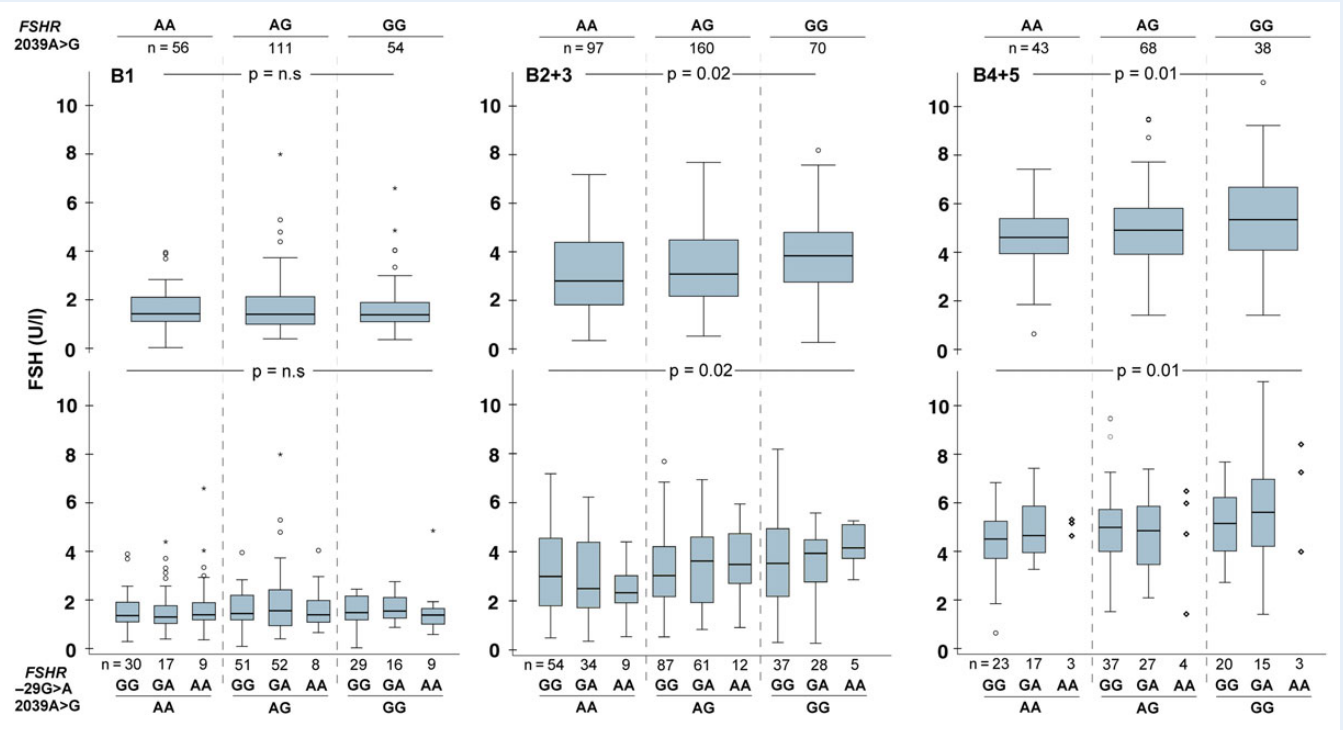

Figure 1 Serum FSH levels according to Tanner subgroups stratified by *FSHR 2039*A>G (upper part) as well as *FSHR* SNP combinations *FSHR* 2039A>G and *FSHR* −29G>A (lower part). Box and whisker plots: medians are marked by thick black lines; boxes contain 50% of the distribution, whiskers outline the range (nonoutliers); dots indicate values more than 1.5 times the interquartile range; asterisks indicate values more than three times the interquartile range. Rhombuses indicate single values if less than five observations were available.

Morphological data indicated reduced maturation of follicles in minor allele carriers of *FSHR* c.-29G>A and *FSHB* c.-211G>T. This may represent the morphological parallel to the previously observed delay of pubertal onset in girls with these genotypes (Hagen *et al.*, 2014). In the present study, the *FSHR* c.2039A>G genotype affected serum FSH levels in girls. The positive effect on serum FSH levels emerged as puberty progressed, with no effect in Tanner stage B1 and significant associations in later stages (B2 + B3 and B4 + B5). The effect became increasingly apparent towards young adolescents (B4 + B5 > B2 + B3), suggesting maturation of feedback regulation along the hypothalamic–pituitary–gonadal (HPG) axis as puberty progresses. Previous studies in adult women showed elevated FSH levels in *FSHR* c.2039A>G minor allele carriers (Perez Mayorga *et al.*, 2000; Yao *et al.*, 2011), supporting the notion of modulated cell response by *FSHR* variants resulting in compensatory higher FSH levels (Gromoll and Simoni, 2005).

Furthermore, comparable with previous studies in adult women (Valkenburg *et al.*, 2009; Boudjenah *et al.*, 2012), we observed that LH levels were also higher in *FSHR* c.2039A>G minor allele carriers. This may be caused by increased GnRH-drive on the common gonadotrophin alpha-subunit compensating for the reduced FSH receptor sensitivity or alteration of the GnRH-pulse frequency/amplitude selectively favouring LH.

Neither follicle growth nor hormone production from small ovarian follicles (AMH) or medium/large follicles (inhibin B) were affected. This indicates that the feedback mechanism fully compensates for the reduced receptor sensitivity of the *FSHR* c.2039A>G minor allele. We speculate that the slightly elevated estradiol levels in minor allele carriers was caused by increased androgen production and aromatase activity induced by elevated LH and FSH, respectively. Indeed, a recent study evaluating follicular fluid hormone levels and granulosa cell gene expression patterns showed that large follicles (>6 mm) in women homozygous for the *FSHR* c.2039A>G minor allele displayed higher *LHR* and *CYP19A1* mRNA levels as well as higher intrafollicular estradiol levels (Borgbo *et al.*, 2015).

When evaluated separately, we found no clear effect of *FSHR* c.-29G>A; however, for the first time we demonstrated a combined effect of the *FSHR* variants c.2039A>G and c.-29G>A on circulating FSH levels. Again, the effect seemed to increase as puberty progressed, and emerged in Tanner stage B2 + B3 and B4 + B5. Previously, a study of 150 adult women failed to show a combined effect of both *FSHR* variants (Desai *et al.*, 2013). Furthermore, a recent GWAS study comprising largely post-menopausal women could not find any association of serum FSH levels with *FSHR* SNPs (Ruth *et al.*, 2016). This may be caused by an insufficient sample size in case of the first study or (as suggested by Simoni and Casarini, 2014 in a recent review) by the advanced age of the included patients increasing the risk of elevated FSH and attenuated feedback regulation due to reduced ovarian reserve.

Our study shows an interesting incongruity concerning the effect of the two *FSHR* SNPs: while *FSHR* c.-29G>A only slightly affected serum FSH levels in young females, *FSHR* c.2039A>G appeared to be an important genetic determinant of serum FSH levels in these girls. As end organ resistance within the HPG axis is usually associated with increased production of the respective hormone, one would expect a pronounced effect of the intragenic variant on downstream outcomes, e.g. follicular

growth, estradiol production or pubertal onset. However, in the same study population as in the present study, *FSHR* c.-29G>A, but not *FSHR* c.2039A>G postponed pubertal onset (defined by thelarche) significantly and *FSHR* c.-29A was more prevalent in girls with delayed puberty (Hagen *et al.*, 2014). Another study revealed a substantial impact of *FSHR* c.-29G>A, but not *FSHR* c.2039A>G, on FSH consumption and ovarian response rate in adult women undergoing controlled ovarian hyperstimulation (Desai *et al.*, 2013). Furthermore, in the present study in the subgroup that underwent TAUS examination *FSHR* c.-29G>A, but not *FSHR* c.2039A>G exerted a moderate negative effect on follicle growth. The reason for the different effect of the *FSHR* SNPs might lie within their distinct functional differences: *FSHR* c.-29G>A attenuates FSHR mRNA transcript levels and thereby the number of receptors present on the cell surface (Nakayama *et al.*, 2006; Desai *et al.*, 2011), whereas *FSHR* c.2039A>G exerts a negative effect on signalling kinetics to FSH stimulation (Nordhoff *et al.*, 2011; Casarini *et al.*, 2014). We speculate that the negative impact of *FSHR* c.2039A>G is fully compensated by increasing FSH levels, while the negative impact *FSHR* c.-29G>A is not sufficiently compensated and thus affects follicle growth and pubertal onset.

FSHB c.-211 G>T was strongly associated with serum LH, but not FSH levels. Again, high LH levels might be caused by increased GnRH-drive on the common gonadotrophin alpha-subunit compensating in this case for the reduced FSH net production or may represent alteration of the GnRH-pulse frequency/amplitude selectively favouring LH. An association between *FSHB* c.-211 G>T and LH has previously been observed in adult women (Schüring *et al.*, 2013). In further support of our findings, a recent GWA study identified a variant in the close vicinity of *FSHB* c.-211 G>T altering gonadotrophin levels (Ruth *et al.*, 2016).

Due to low minor allele frequency and hence insufficient group size, we were not able to contribute substantially to the conflicting results in adult women concerning the influence of *FSHB* c.-211 G>T on circulating FSH levels (La Marca *et al.*, 2013; Schüring *et al.*, 2013). However, with regard to recent GWA studies pointing to a contributory role of this genetic variant (or those in the vicinity) to the polycystic ovary syndrome (PCOS) phenotype (Day *et al.*, 2015a; Hayes *et al.*, 2015; Ruth *et al.*, 2016), it is noteworthy that we observed the serum LH/FSH ratio, the biochemical hallmark PCOS, to be positively associated with *FSHB* c.-211 G>T. The association was primarily driven by increased LH levels. Given the limitation that in case a girl had encountered menarche no information was available about the exact day of menstrual cycle when the blood sample was drawn, these findings should be considered with caution particularly in the group of young adolescents. However, noticeably, we found a significant association even in prepubertal girls, potentially pointing to a LH/FSH crosstalk within the HGP axis even in the presence of low activity of the HPG axis. Assuming a variant-mediated reduced FSH receptor sensitivity (*FSHR* c.-29G>A) and reduced FSHB net production (*FSHB* c.-211G>T), the observed negative associations between minor allele counts (cumulated) and the number of large ovarian follicles (as well as effects on the small to large follicle ratio) suggest an attenuated FSH signalling reducing follicle growth. Although puberty relies on central reactivation of the HPG axis at the top level, i.e. hypothalamus, including gene products of *KISS1, TAC3, GNRH1* etc. (Perry *et al.*, 2015), concerted action of all three integral parts of the axis is critical for full activation of the axis. Thus, inactivating mutations at each level severely impair pubertal onset and progression (Huhtaniemi and Themmen, 2005; Abreu *et al.*, 2013; Quaynor *et al.*, 2013). With regard to this, we speculate that the observed reduced large follicle count and altered follicle size ratio affects estradiol production during pubertal transition and hence the timing of thelarche. This would represent the morphological parallel to our previous observations of postponed pubertal onset in girls with reduced FSH receptor sensitivity (*FSHR* c.-29G>A) and FSH production (*FSHB* c.-211G>T) (Hagen *et al.*, 2014).

The hypothesis would be backed up by associations with products from large follicles (estradiol and inhibin B). However, due to low concentrations and insensitive immunoassays, the majority of prepubertal girls had estradiol and inhibin B levels below the detection limit of the assays (Hagen *et al.*, 2010). Thus, we cannot exclude a subtle but biological relevant effect of the genotypes in prepubertal girls (Day *et al.* 2015a,b; Hayes *et al.*, 2015).

Circulating AMH in girls reflects the number of small and medium antral follicles (Hagen *et al.*, 2015). In a subpopulation of the present study, we had previously observed low AMH levels in prepubertal girls with high FSH production and high FSH receptor sensitivity (*FSHB* c.-211GG + *FSHR* c.2039AA) (Hagen *et al.*, 2013), and speculated that the number of AMH producing follicles would be reduced due to increased FSH-induced follicle growth. However, the absolute number of small and medium follicles (the antral follicle count, AFC) was not associated with the genotypes in the present study, neither when adjusted for Tanner stages nor in Tanner subgroup analysis. This probably explains the lack of associations with circulating AMH levels in this larger study enabling more detailed analyses. With regard to the observations in the group of young adolescents (Tanner B4 + B5), most likely to resemble adult women, previous studies in adult women showed comparable findings (Mohiyiddeen *et al.*, 2012; La Marca *et al.*, 2013).

The majority of prepubertal girls in the present study were above 8 years. Therefore, effects of genetic variation in young prepubertal girls cannot be evaluated in this study. Unfortunately, we have no information about the exact day of menstrual cycle the blood samples were drawn from the subgroup of girls with menarche. However, the risk of samples being collected at the time of ovulation is equal across the genotype groups.

In conclusion, *FSHR* c.2039A>G was associated with FSH, LH and estradiol levels, and for the first time, we were able to show a combined effect of two FSH receptor variants on serum FSH levels. Morphological data suggest impaired FSH-induced maturation of follicles in minor allele carriers of *FSHB* c.-211G>T and *FSHR* c.-29G>A. This may explain previous findings of delayed pubertal onset in these girls.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgments

The authors would like to thank the participating children and all colleagues who were involved in The COPENHAGEN Puberty Study and the Copenhagen Mother–Child Cohort. Special thanks go to staff of the hormone laboratory and the genetic laboratory of the Department of Growth and Reproduction, Rigshospitalet for their expert technical assistance.

Authors' roles

A.S.B.: data analysis and manuscript drafting. C.P.H.: data analysis, manuscript drafting and execution. K.A.: data analysis, execution and critical discussion. K.M.M.: study design, execution and critical discussion. A.J.: study design, execution and critical discussion. All authors approved the final manuscript.

Funding

Funding was provided by the Danish Agency for Science, Technology and Innovation (09-067180), Danish Ministry of the Environment, CeHoS (MST-621-00065), Capital Region of Denmark (December 2011), Ministry of Higher Education and Science (DFF—1331-00113) and EDMaRC (Danish Ministry of Health). A.S.B. was funded from December 2015 by ReproUnion (EU Interreg øresund-Kattegat-Skagerrak).

Conflict of interest

None declared.

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