Genetic Variation of Follicle-Stimulating Hormone Action Is Associated With Age at Testicular Growth in Boys

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Context: Although genetic factors play a pivotal role in male pubertal timing, genome-wide association studies have identified only a few loci. Genetic variation of follicle-stimulating hormone (FSH) action affects adult reproductive parameters and female pubertal timing.

Objective: To investigate whether genetic variation affecting FSH action is associated with onset of puberty in boys.

Design: Cross-sectional and longitudinal study of two cohorts of healthy boys.

Setting: This was a population-based study.

Patients or Other Participants: Danish (n = 1130) and Chilean (n = 424) boys were followed through puberty and genotyped for *FSHB* c.-211G>T, *FSHR* c.-29A>G, and *FSHR* c.2039G>A.

Main Outcome Measures: Clinical pubertal staging including orchidometry, anthropometry, and serum gonadotropin levels.

Results: Although the cohorts differed markedly (*e.g.*, body composition and genotype frequencies), genetic variation affecting FSH production (*FSHB* c.-211G>T) was associated with age at pubertal onset, as assessed by testicular enlargement, in both cohorts. The effect appeared further modified by co-existence of genetic variation affecting FSH sensitivity (*FSHR* c.-29G>A): After correcting for body mass index (BMI), boys with a ligand-receptor variant combination resulting in weak FSH action (*i.e.*, *FSHB* c.-211GT/TT and *FSHR* c.-29AA) entered puberty 0.64 years [95% confidence interval (CI), 0.12 to 1.17 years; Denmark] and 0.94 years (95% CI, 0.00 to 1.88 years; Chile) later than boys with the most effective FSH action. Effects explained 1.7% (Denmark) and 1.5% (Chile) of the variance. In addition, BMI *z* score was negatively associated with pubertal timing ($\beta = -0.35$ years in both cohorts), explaining 17.2% (Denmark) and 7.2% (Chile) of the variance.

Conclusion: In two ethnically distinct populations, we independently identified an association of two genetic loci with male pubertal timing. **(*J Clin Endocrinol Metab* 102: 1740–1749, 2017)**

Although age at pubertal onset exhibits a remarkable interindividual as well as ethnic variation (1), regulators of pubertal timing remain to be elucidated. This variation stems from nutritional, environmental, socioeconomic, and genetic factors (2), and twin studies suggest that the latter accounts for

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Abbreviations: BMI, body mass index; CI, confidence interval; FSH, follicle-stimulating hormone; GWAS, genome-wide association study; LH, luteinizing hormone; SNP, single nucleotide polymorphism.

more than half of the phenotypic variation of pubertal onset (3).

Recent large-scale, genome-wide association studies (GWASs), relying on recall of pubertal events (*i.e.*, age at menarche in girls and age at voice break in boys), identified genetic loci associated with pubertal timing (106 loci in girls and 9 loci in boys) (4, 5). Results indicated a shared genetic etiology of pubertal timing in boys and girls. However, these studies could explain only a small percentage of the phenotypic variation of pubertal timing. Particularly in boys, only nine loci have been identified. Important cofactors [*e.g.*, body mass index (BMI)] (6) were not taken into account because they are usually not available in GWASs.

The onset of puberty is characterized by the central reactivation of the hypothalamic-pituitary-gonadal axis after a period of relative quiescence during childhood. In boys, testicular enlargement is the clinical hallmark of pubertal onset (7). Follicle-stimulating hormone (FSH) stimulates Sertoli cell proliferation and maturation, drives testicular enlargement during pubertal development, and maintains spermatogenesis synergistically with testosterone in the adult testis (8, 9). Genetic variation of *FSHB* and *FSHR*, coding for the specific FSH β-subunit and the FSH receptor, is known to alter FSH action via reduced *FSHB* promoter activity (*FSHB* c.-211G>T) (10), reduced FSH receptor cell surface expression (*FSHR* c.-29A>G) (11), and reduced FSH signal transduction (*FSHR* c.2039G>A) (12). Consequently, these variants have been shown to be associated with reproductive function in adult men (*i.e.*, serum FSH levels, testis size, and sperm count) (13–15). In women, where FSH promotes granulosa cell proliferation and differentiation as well as estradiol synthesis, genetic variation altering FSH action has been associated with pubertal and menopausal timing (16, 17), gonadotropin levels (18–20), and susceptibility for polycystic ovary syndrome (21).

In this study, we followed healthy boys from two populations—Danish and Chilean—longitudinally through puberty and we report an association of distinct, common genetic variants (*i.e.*, *FSHB* c.-211G>T and *FSHR* c.-29A>G) with clinical signs of pubertal onset in boys as determined by testicular enlargements ≥4 mL.

Materials and Methods

Study populations

Healthy boys between 5 and 19 years old with clinical pubertal staging, BMI data, and *FSHB* c.-211G>T and/or both *FSHR* genotypes (*i.e.*, *FSHR* c.-29A>G, *FSHR* c.-2039G>A) were included in the current study.

Healthy Danish boys

Participants were recruited as part of three population-based cohort studies of healthy Danish children and adolescents. The Copenhagen Puberty Study [ClinicalTrials.gov ID: NCT01411527; detailed information about the study has been given previously (22)] is a combined cross-sectional (cohort A) and longitudinal study (cohort B) that has been conducted at 10 schools in the Copenhagen area between 2006 and 2014. A total of 3101 boys were invited to join the study, of whom 767 boys were examined (overall participation rate, 25%). The Copenhagen Mother-Child Cohort (cohort C; detailed information about the study has been given previously) (23) is based on a birth cohort of Danish children born at three hospitals in the Copenhagen area between 1997 and 2003. The children were examined at several time points during infancy and childhood, and 1518 peripubertal children (846 boys) agreed to participate in a longitudinal study with annual examinations (participation rate, 43%). All children and parents received written information, and informed consent was obtained from all participants. A total of 1130 Danish boys from the three cohorts were included in the current study, including a total of 3025 clinical examinations. Data on serum gonadotropin levels were available for 1094 boys from 1679 examinations.

Healthy Chilean boys

Participants were recruited as part of the Growth and Obesity Chilean Cohort Study (24). All children attending the Chilean National Nursery School Council Program from the southeast area of Santiago, Chile, in 2006 and who were singletons, gestational age 37 to 42 weeks, birth weight ≥2500 g and ≤4500 g, and had no physical or psychological conditions that could severely affect growth were eligible for the study. Children were recruited at age 3.0 to 4.9 years. Of these, 1195 (85%) agreed to participate. No relevant differences in age and birth or current anthropometric characteristics at inclusion between participants and nonparticipants were noted. Thereafter, annual evaluations including anthropometry and pubertal staging were conducted. Since 2009, biannual evaluations were conducted. Written informed consent was obtained from all parents or guardians of the children. A total of 424 Chilean boys were included in the current study, including a total of 3032 clinical examinations.

Clinical examination

Examinations were performed by trained professionals in both countries. In Denmark, three pediatric endocrinologists examined the children in cohorts A and B, and three pediatric endocrinologists examined those in cohort C. Eight young pediatricians were trained by experienced pediatric endocrinologists (A.J., K.M.M.) to evaluate pubertal development. In Chile, annual standardization was carried out between a pediatric endocrinologist (V.M.) and a male dietitian (trained by V.M.; Cohen κ = 0.8). Clinical examinations included pubertal staging of testicular development according to the Tanner classification and testicular volume (7). Testicular volume ≥4 mL (at least one testis) was considered to be a marker of onset of puberty.

Genotyping

Peripheral blood (0.2 mL preserved in EDTA) was used for isolation of genomic DNA and genotyping was performed with KASP (LGC Genomics, Hoddesdon, UK) genotyping assays, as described previously (16). *FSHB* c.-211G>T (rs10835638), *FSHR* c.-29G>A (rs1394205), and *FSHR* c.2039A>G (rs6166) genotypes were available in 1125, 1128, and 1128

Danish boys and in 424, 423, 424 Chilean boys, respectively. All three genotypes were available in 1123 Danish and 423 Chilean boys.

Reproductive hormone assays (Danish samples only)

All nonfasting blood samples from Danish boys were drawn between 8:00 AM and 2:00 PM from an antecubital vein, then clotted and centrifuged; serum was stored at −20°C until hormone analyses were performed. Serum levels of FSH and luteinizing hormone (LH) were measured by time-resolved immunofluorometric assays (Delfia; PerkinElmer, Boston, MA) with detection limits of 0.06 and 0.05 IU/L, respectively. Intra- and interassay coefficients of variation were <5%.

Statistical analyses

To estimate the mean age [95% confidence interval (CI)] at pubertal onset, we performed probit analyses (25) (proc lifereg; SAS Institute, Cary, NC) integrating left-, right-, and interval-censored observations and testis volume ≥4 mL, with yes/no as the binomial response variable. Longitudinal data of boys entering puberty during follow-up (Chilean cohort, n = 309; Danish cohort, n = 252) were included in the probit analyses (interval censored data). If a boy had not entered puberty at his last examination (or at his only examination if seen once), his age at examination was used as right-censored data (Chilean cohort, n = 109; Danish cohort, n = 471). If a boy had entered puberty at his first examination, his age at examination was used as left-censored data (Chilean cohort, n = 6; Danish cohort, n = 407). Both cohorts were standardized by applying World Health Organization age-specific BMI scores (BMI *z* scores) (26). If longitudinal data were available (Chilean cohort, n = 424; Danish cohort, n = 566) a mean of all individual BMI *z* scores was calculated. In probit analyses, age at pubertal onset was adjusted for BMI *z* score. In joint analysis, we further adjusted for underlying hidden factors as country of origin (*i.e.*, Denmark or Chile). Interactions between categorical variables were tested via the lifereg MODEL statement (SAS Institute).

To evaluate whether BMI *z* score differed significantly between the two cohorts, we performed a Student *t* test. To evaluate whether individual BMI *z* scores were stable throughout puberty, we performed a paired sample *t* test comparing mean pre- and postpubertal individual BMI *z* scores (interval-censored data only). To evaluate the effect of BMI *z* score on age at pubertal onset, we adjusted the probit analysis for BMI *z* score as a continuous variable.

We performed χ^2 tests to evaluate whether genotype frequencies differed significantly between the cohorts. When evaluating genotype effects, we used three models: additive, recessive, and dominant. The additive model assumes a cumulative allele effect in a dose-dependent manner. This model was also used to assess the effect of genotype combinations. In the recessive model, effects were expected only in the minor-allele homozygotes compared with pooled wild-type and heterozygotes. In the dominant model, effects were expected in wild-type allele homozygotes compared with pooled minor-allele homozygotes and heterozygotes.

To evaluate the effect of the genetic variants on serum gonadotropin levels, we performed Mann Whitney *U* tests (wild-type homozygotes vs minor-allele carriers) for three groups according to pubertal development: prepubertal, testicular volume <4 mL; midpubertal, testicular volume ≥4 mL and <12 mL; and late pubertal, testicular volume ≥12 mL). Gonadotropin levels below the detection limit were assigned 0.5 times the detection limit. If several blood samples throughout different pubertal stages were available (n = 154 boys), observations were grouped by pubertal stage. If multiple observations within a specific pubertal stage were available, a mean value was calculated and introduced into the model like the single-occasion observation.

To estimate effect of the genetic variants as well as BMI *z* score on age at pubertal onset, 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 or BMI *z* score was included as an explanatory variable. In the second model, we estimated the variance in a probit analysis in which no explanatory variable was included. The difference between the two variances is due to the genetic variants or BMI *z* score included in the model. $P \leq 0.05$ was considered statistically significant.

Ethical considerations

The Copenhagen Puberty Study and The Copenhagen Mother-Child Cohort were approved by the ethical committee of 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, and 2006-41-7251). The Growth and Obesity Chilean Cohort Study protocol was approved by the institutional review board of the Institute of Nutrition and Food Technology of the University of Chile (No. 14). All experiments were performed in accordance with the relevant guidelines and regulations.

Results

Age at pubertal onset, as defined by testicular enlargement2 ≥4 mL; BMI *z* scores; and genotype frequencies differed significantly between the Danish and Chilean cohorts (Tables 1 and 2). The mean age at pubertal onset was 11.7 and 11.0 years in Danish and Chilean boys, respectively ($P < 0.001$). Chilean boys exhibited higher BMI *z* scores compared with Danish boys ($P < 0.001$; Table 1). Individual BMI *z* scores remained stable throughout pubertal transition (Fig. 1) and pre- and postpubertal values did not differ significantly between Danish and Chilean boys ($P = 0.08$ and $P = 0.06$, respectively; Fig. 1). Mean individual BMI *z* scores approximated a linear negative correlation with age at pubertal onset in both cohorts ($\beta = -0.35$ years in both cohorts; 95% CI for Denmark cohort, −0.43 to −0.27 years; and for Chilean cohort, −0.48 to −0.21 years). After correction for BMI *z* score, age at pubertal onset between the cohorts still differed significantly (Table 1) while country of origin (BMI *z* score corrected) accounted for a difference of 0.40 years between the cohorts (95% CI, 0.22 to 0.58 years).

When analyzing the effect of genetic variants regardless of BMI, we observed a significant effect of the

Table 1. Basic Characteristics of the Danish and the Chilean Cohorts

Characteristic	Danish Boys (n = 1130)	Chilean Boys (n = 424)	*P*
Age at pubertal onset	11.67 (11.59 to 11.76)	11.00 (10.83 to 11.16)	< 0.001
Age at pubertal onset (adjusted for BMI *z* score)[a]	11.73 (11.62 to 11.83)	11.33 (11.18 to 11.48)	< 0.001
Standardized BMI *z* score[a,b]	0.16 ± 0.97 0.12 (−1.49 to 1.78)	1.12 ± 1.20 1.09 (−0.80 to 3.06)	< 0.001

Values are mean (95% CI), year.

[a]World Health Organization growth reference 5 to 19 years old (26).

[b]Data presented as mean ± standard deviation and median (fifth to 95th percentiles).

FSHB c.-211G>T on age at pubertal onset in the Danish cohort (dominant model, $P = 0.02$) but not the Chilean cohort (Table 3). Adjusting for BMI *z* score increased significance in the Danish cohort (dominant model, $P = 0.008$) and showed a similar but nonsignificant trend in the Chilean cohort (dominant model, $P = 0.07$). After adjustment for BMI *z* score, we observed a significant association of *FSHB* c.-211G>T with age at pubertal onset in both cohorts in the additive model: Danish, $P = 0.03$, T-allele effect = 0.16 years; Chilean, $P = 0.04$, T-allele effect = 0.39 years. A joint analysis of both cohorts revealed a significant effect of *FSHB* c.-211G>T and a nonsignificant trend for *FSHR* c.-29G>A on age at pubertal onset (adjusted for mean BMI *z* score as well as country of origin; Table 4).

Based on the recessive effect of *FSHR* c.-29G>A and the dominant effect of *FSHB* c.-211G>T, we developed a combined genetic model of FSH action [*FSHB* c.-211G>T and *FSHR* c.-29G>A; Fig. 2(d)] that revealed significant effect on age at pubertal onset: additive model, Danish cohort: $P = 0.008$, $\beta = 0.11$ years; Chilean cohort: $P = 0.02$, $\beta = 0.22$ years (adjusted for BMI *z* score); joint analysis $P < 0.001$, $\beta = 0.15$ years [adjusted for BMI *z* score and country of origin; Table 3 and Fig. 2(a–c)]. The "extreme" groups (*i.e.*, *FSHB* c.-211GG and *FSHR* c.-29GG/GA vs *FSHB* c.-211GT/TT and *FSHR* c.-29AA) entered puberty with a difference of 0.64 years [95% CI, 0.12 to 1.17 years; adjusted for BMI *z* score, Denmark; Fig. 2(a)] and 0.94 years [95% CI, 0.00 to 1.88 years; adjusted for BMI *z* score, Chile; Fig. 2(b)] [both cohorts combined, 0.81 years; 95% CI, 0.26 to 1.32; adjusted for BMI *z* score and country of origin; Fig. 2(c)]. *FSHB* c.-211G>T did not interact significantly with *FSHR* c.-29G>A (Denmark, $P = 0.06$, and Chile, $P = 0.61$, adjusted for BMI *z* score; joint analysis, $P = 0.17$, adjusted for BMI *z* score and country of origin). In the group of *FSHB* c.-211G>T minor-allele carriers (*FSHB* c.-211GT and TT), we observed significant effect on age at pubertal onset in *FSHR* c.-29G>A minor-allele homozygotes (*FSHR* c.-29AA) compared with wild-type carriers [*FSHR* c.-29GG and GA; $\beta = 0.54$ years (95% CI, 0.04 to 1.04 years); $P = 0.03$, adjusted for BMI *z* score and country of origin]. *FSHR* c.2039A>G did not exert an effect on age at pubertal onset, neither alone nor in combination (Table 3).

We estimated that *FSHB* c.-211G>T explained 1.0% of the variance in age at pubertal onset in the Danish cohort and 0.7% in the Chilean cohort. The combined genetic model of FSH action (*FSHB* c.-211G>T and *FSHR* c.-29G>A) accounted for 1.7% of the variance in the Danish cohort and 1.5% in the Chilean cohort. In an independent analysis, BMI *z* score explained 17.2% of the variance in age at pubertal onset in the Danish cohort and 7.2% in the Chilean cohort.

Analysis of gonadotropin levels did not reveal any significant alteration between genotype groups [data not

Table 2. Genotype and Allele Frequencies in the Danish and the Chilean Cohorts

	Danish Boys (n = 1130)			Chilean Boys (n = 424)			
Frequencies, %	**WW**	**WM**	**MM**	**WW**	**WM**	**MM**	***P***
Genotype							
FSHB c.-211G>T	67.2	29.7	3.1	80.0	19.3	0.7	< 0.001
FSHR c.-29G>A	52.3	39.8	7.7	26.5	51.2	22.2	< 0.001
FSHR c.2039A>G	29.0	50.0	20.8	43.9	42.7	13.4	< 0.001
Allele	W		M	W		M	
FSHB c.-211G>T	82		18	89.6		10.4	< 0.001
FSHR c.-29G>A	72.3		27.7	52.1		47.9	< 0.001
FSHR c.2039A>G	54.1		45.9	65.2		34.8	< 0.001

Abbreviations: M, minor allele; W, wild-type allele.

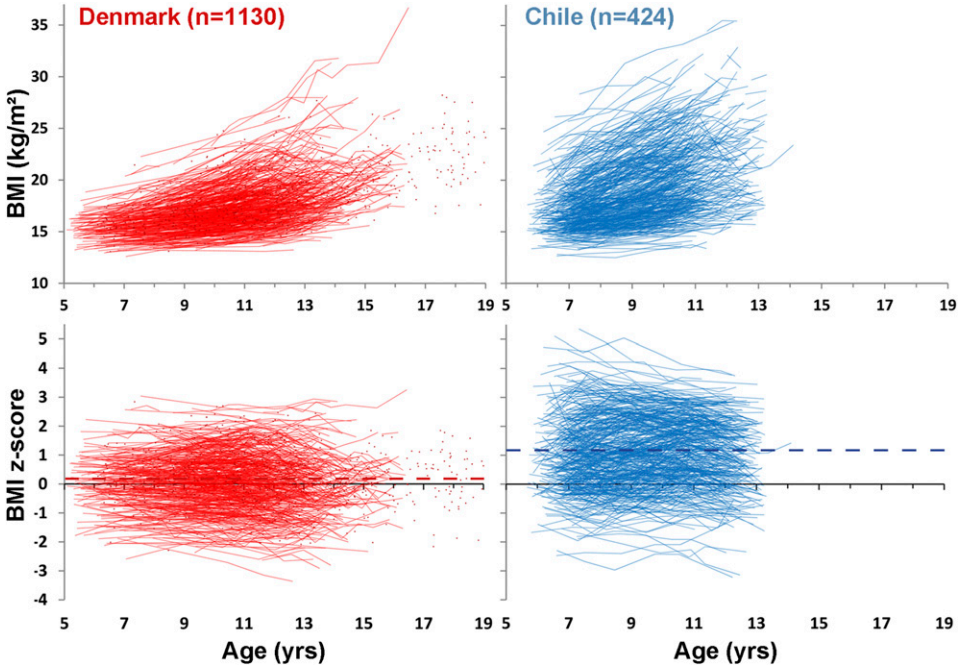

Figure 1. BMI and BMI *z* scores of the Danish and the Chilean cohort. Individual BMI and BMI *z* scores of Danish (red, n = 1130) and Chilean (blue, n = 424) boys according to the World Health Organization growth reference (26) in relation to age. Dots represent cross-sectional data. Lines represent consecutive measurements in a single boy (longitudinal data). Dashed lines represent median BMI *z* score of the cohorts. The Chilean cohort displays significantly higher BMI *z* scores compared with the Danish cohort.

shown; *FSHB* c.-211G>T (Supplemental Table 1)]. However, there was a trend for lower FSH levels in prepubertal *FSHB* c.-211G>T minor-allele carriers compared with wild-type homozygotes and the FSH/LH ratio was significantly decreased in minor-allele carriers in prepubertal and midpubertal stages.

Discussion

In this study comprising 1554 healthy boys from two independent populations, *FSHB* c.-211G>T was associated with age at testicular enlargement. The effect appeared further modified by *FSHR* c.-29G>A. Despite largely different genotype frequencies between the Danish and the Chilean boys, the effect was observed in both cohorts and the effect estimates were of similar magnitude. This is, to our knowledge, the largest effect of single nucleotide polymorphism (SNP) variants on age at male pubertal onset reported to date.

The observed effect of the two genetic variants follows a defined biological mechanism of reduced *FSHB* promoter activity via an altered transcription factor binding site (*FSHB* c.-211G>T) (10, 27) resulting in reduced FSH production (15) and reduced cell-surface expression of the FSH receptor (*FSHR* c.-29G>A) (11). FSH stimulates Sertoli cell proliferation and lengthening of seminiferous tubules, leading to testis growth (28, 29), the clinical hallmark of male pubertal onset and actual proxy for pubertal onset in our study.

The effect of *FSHB* c.-211G>T on age at pubertal onset (0.23 years per T allele) was twice as large as the top signal (*LIN28B*) in a recent GWAS based on self-reported age at voice break (5). In addition, the combination of *FSHB* c.-211G>T and *FSHR* c.-29G>A appeared to further modulate age at pubertal onset in our study. Our observations might point to a role of ligand-receptor interplay: Although the two variants did not interact significantly when tested in the present joint analysis, a clear modulative effect of *FSHR* c.-29G>A was apparent in *FSHB* c.-211G>T minor-allele carriers. In the proposed combined genetic model, boys with ligand-receptor variant combination resulting in weak FSH action (*i.e.*, *FSHB* c.-211GT/TT and *FSHR* c.-29AA) entered puberty substantially later than boys with the most effective FSH action (*i.e.*, *FSHB* c.-211GG and *FSHR* c.-29GG/GA). A comparable potentiating effect of the two SNPs has been observed in girls relative to pubertal onset (16) and in adult men relative to testicular function (13). Interestingly, later self-reported puberty is associated with poor semen quality (30), which we speculate could be caused by genetically compromised FSH action.

The variance of pubertal onset in Danish and Chilean boys explained by *FSHB* c.-211G>T (1.0% and 0.7%, respectively) and the genetic model of FSH action (*FSHB* c.-211G>T and *FSHR* c.-29G>A, 1.7% and 1.5%, respectively) is noteworthy. Comparable estimates are not available from other male cohorts; however, in the largest GWAS on female pubertal timing, the top 123 SNPs

Table 3. Age of Pubertal Onset Stratified by *FSHB*/*FSHR* Genotypes and the Combined Genetic Model in the Danish and Chilean Cohorts

				Noncorrected[a]					Corrected for BMI *z* Score[a]				
	SNP	Genotype	No.	Age at Pubertal Onset, y	95% CI	Additive, y[b]	Recessive Model, y[b]	Dominant Model, y[b]	Age at Pubertal Onset, y	95% CI	Additive Model, y[b]	Recessive Model, y[b]	Dominant Model, y[b]
Danish cohort (n = 1130)	*FSHB* c.-211G>T	GG	756	11.6	11.5–11.7	0.06,	0.47,	0.02,[c]	11.6	11.5–11.7	0.03,[c]	0.50,	0.008,[d]
		GT	334	11.8	11.7–12.0	0.15	−0.18	0.21	11.9	11.7–12.0	0.16	−0.16	0.23
		TT	35	11.5	11.0–12.0	(−0.01 to 0.30)	(−0.67 to 0.31)	(0.04–0.39)	11.6	11.1–12.0	(0.01–0.30)	(−0.61 to 0.30)	(0.0–0.39)
	FSHR c.-29G>A	GG	591	11.7	11.6–11.8	0.83,	0.58,	0.57,	11.7	11.6–11.8	0.65,	0.78,	0.47,
		GA	450	11.6	11.5–11.8	−0.01	0.09	−0.05	11.7	11.5–11.8	−0.03	0.04	−0.06
		AA	87	11.8	11.4–12.1	(−0.15 to 0.12)	(−0.23 to 0.41)	(−0.22 to 0.12)	11.7	11.5–12.0	(−0.15 to 0.10)	(−0.26 to 0.34)	(−0.22 to 0.10)
	FSHR c.2039A>G	AA	328	11.6	11.5–11.8	0.85,	0.23,	0.43,	11.7	11.5–11.8	0.97,	0.28,	0.36,
		AG	565	11.8	11.6–11.9	−0.01	−0.12	0.07	11.8	11.7–11.9	0.00	−0.11	0.08
		GG	235	11.6	11.4–11.8	(−0.13 to 0.11)	(−0.33 to 0.08)	(−0.11 to 0.26)	11.6	11.5–11.8	(−0.11 to 0.11)	(−0.29 to 0.08)	(−0.09 to 0.25)
	FSHB c.-211G>T	***FSHR* c.-29G>A**											
		GG and **GG/AG**	694	11.6	11.5–11.7	0.01,[c]			11.6	11.5–11.7	0.008,[d]		
		GG and **AA**	60	11.5	11.1–11.9	0.11			11.5	11.1–11.8	0.11		
		GT/TT and **GG/AG**	342	11.8	11.6–11.9	(0.02–0.19)			11.8	11.7–12.0	(0.03–0.19)		
		GT/TT and **AA**	27	12.3	11.7–12.8				12.3	11.8–12.8			
Chilean cohort (n = 424)	*FSHB* c.-211G>T	GG	339	10.9	10.8–11.1	0.09,	0.14,	0.14,	11.3	11.1–11.5	0.04,[c]	0.16,	0.07,
		GT	82	11.2	10.8–11.6	0.34	1.81	0.31	11.6	11.2–12.0	0.39	1.65	0.37
		TT	3	12.8	10.4–15.2	(−0.05 to 0.73)	(−0.57 to 4.18)	(−0.10 to 0.72)	13.0	10.8–15.3	(0.01–0.77)	(−0.63 to 3.9)	(−0.02 to 0.77)
	FSHR c.-29G>A	GG	112	11.0	10.7–11.3	0.42,	0.17,	0.97,	11.3	11.0–11.7	0.23,	0.11,	0.72,
		GA	217	10.9	10.7–11.1	0.10	0.28	−0.01	11.3	11.0–11.6	0.14	0.31	0.06
		AA	94	11.2	10.9–11.6	(−0.14 to 0.33)	(−0.12 to 0.67)	(−0.38 to 0.36)	11.6	11.2–12.0	(−0.09 to 0.37)	(−0.07 to 0.69)	(−0.29 to 0.42)
	FSHR c.2039A>G	AA	186	11.0	10.8–11.3	0.66,	0.48,	0.90,	11.4	11.1–11.7	0.57,	0.32	0.91,
		AG	181	11.0	10.8–11.3	−0.05	−0.17	−0.02	11.4	11.1–11.7	−0.07	−0.23	−0.02
		GG	57	10.8	10.4–11.3	(−0.29 to 0.18)	(−0.65 to 0.30)	(−0.35 to 0.31)	11.2	10.7–11.6	(−0.29 to 0.16)	(−0.69 to 0.23)	(−0.34 to 0.30)
	FSHB c.-211G>T	***FSHR* c.-29G>A**											
		GG and **GG/AG**	259	10.9	10.7–11.1	0.06,			11.2	11.0–11.5	0.02,[c]		
		GG and **AA**	80	11.1	10.7–11.5	0.18			11.5	11.1–11.9	0.22		
		GT/TT and **GG/AG**	70	11.1	10.7–11.5	(−0.01 to 0.34)			11.6	11.1–12.0	(0.04–0.40)		
		GT/TT and **AA**	14	11.9	10.9–12.8				12.2	11.2–13.1			

Boldface indicates that GG/AG and AA refers to *FSHR* c.-29G>A. GG and GT/TT refers to *FSHB* c.-211G>T.

[a] Additive model: WW vs WM vs MM, recessive model WW+WM vs MM, dominant model WW vs WM+MM.

[b] Model, *P* value, effect size (95% CI).

[c] $P < 0.05$.

[d] $P < 0.01$.

together explained 2.7% of the phenotypic variation of age at menarche. Apart from the effect at population level, the distinct genetic variant combination of *FSHB* c.-211G>T and *FSHR* c.-29G>A may lead to a substantial delay of pubertal onset by several months in a single individual. Studies of male patients with constitutional delay of growth and puberty are needed to elucidate whether genetic variation of FSH action plays a part in the etiology of this condition.

Age at pubertal onset varies greatly between populations. We observed later pubertal onset and higher minor-allele frequency of *FSHB* c.-211 G>T in Danish compared with Chilean boys. The same pattern is observed in Chinese boys with low minor-allele frequency (2.9% in the Han Chinese in Beijing, China, population) (31) and earlier age at pubertal onset (*i.e.*, 10.55 years) (32). Thus, part of the ethnic difference in age at pubertal onset may be explained by different distributions of genotypes affecting FSH action.

The large effect of the *FSHB* locus in our present study stands somewhat in contrast to the GWAS by Day *et al.* (5) in which *FSHB* was not associated with age at voice break (n ≈ 56,000). Age at voice break represents a marker of midpuberty and two explanations appear plausible. Primarily, testicular enlargement is directly dependent on FSH action, whereas voice break is driven by testosterone, suggesting an alternative biological pathway (8, 9, 33). Recent studies on female pubertal timing might serve as an example for the differential impact of genetic variants: Whereas *FSHB* c.-211 G>T was significantly associated with age at thelarche, exhibiting a large effect on this event (16), a GWAS observed only a minor impact on age at menarche (4). Another explanation is that the well-conducted study by Day *et al.* (5) might have been underpowered to detect effects of loci with low minor-allele frequencies, because there were fewer participants than in previous GWASs on girls (4) and because self-reported age at voice break was assessed in rather large intervals of 2 years.

Furthermore, the GWAS approach assumes a rigid system of single genetic variations separately influencing a distinct outcome. However, the hypothalamic-pituitary-gonadal axis represents a dynamic feedback system including dynamic ligand-receptor interaction (34). Thus, particularly the potential modulative effect of the FSH-receptor variant (*FSHR* c.-29A) might not reach genome-wide significance in GWAS, because its effect emerges only in case of reduced FSH production (*FSHB* c.-211T). Given the low *FSHB* c.-211G>T minor-allele frequency, the effect of this ligand receptor interplay might be overseen in GWASs.

Table 4. Age of Pubertal Onset Stratified by *FSHB*/*FSHR* Genotypes and the Combined Genetic Model in Joint Analysis

				Corrected for BMI *z* Score[a]					Corrected for BMI *z* score and Country of Origin[a]				
	SNP	Genotype	No.	Age at Pubertal Onset, y	95% CI	Additive Model, y[b]	Recessive Model, y[b]	Dominant Model, y[b]	Age at Pubertal Onset, y	95% CI	Additive Model, *P* for Effect (95% CI), y	Recessive Model, *P* for Effect (95% CI), y	Dominant Model, *P* for Effect (95% CI), y
Joint analysis (n = 1554)	*FSHB* c.-211G>T	GG	1095	11.5	11.4–11.6	0.0007,[c] (0.12–0.44)	0.80, (−0.50 to 0.65)	0.0002,[c] (0.16–0.52)	11.6	11.5–11.8	0.005,[d] 0.23 (0.07–0.39)	0.95, −0.02 (−0.58 to 0.55)	0.002,[d] 0.29 (0.11–0.47)
		GT	416	11.9	11.7–12.0	0.28	0.07	0.34	11.9	11.8–12.1			
		TT	38	11.7	11.1–12.2				11.7	11.2–12.3			
	FSHR c.-29G>A	GG	703	11.7	11.5–11.8	0.67, −0.03 (−0.15 to 0.09)	0.37, 0.11 (−0.13 to 0.35)	0.22, −0.10 (−0.27 to 0.06)	11.7	11.6–11.9	0.47, 0.04 (−0.08 to 0.17)	0.08, 0.22 (−0.02 to 0.45)	0.80, −0.02 (−0.19 to 0.15)
		GA	667	11.5	11.4–11.6				11.7	11.5–11.8			
		AA	181	11.7	11.5–11.9				11.9	11.7–12.2			
	FSHR c.2039A>G	AA	514	11.5	11.4–11.7	0.82, 0.01 (−0.10 to 0.13)	0.30, −0.11 (−0.32 to 0.10)	0.24, 0.10 (−0.07 to 0.27)	11.7	11.5–11.9	0.72, 0.02 (−0.13 to 0.09)	0.15, −0.15 (−0.36 to 0.06)	0.53, 0.05 (−0.11 to 0.22)
		AG	746	11.7	11.6–11.8				11.8	11.7–11.9			
		GG	292	11.5	11.3–11.7				11.6	11.4–11.8			
	FSHB c.-211G>T	***FSHR* c.-29G>A**											
		GG and **GG/AG**	953	11.5	11.4–11.6	0.0001,[c] 0.17 (0.08–0.26)			11.6	11.5–11.7	0.0004,[c] 0.15 (0.07–0.24)		
		GG and **AA**	140	11.6	11.3–11.8				11.8	11.5–12.0			
		GT/TT and **GG/AG**	412	11.8	11.6–12.0				11.9	11.7–12.0			
		GT/TT and **AA**	41	12.3	11.8–12.8				12.4	11.9–12.9			

Boldface indicates that GG/AG and AA refers to *FSHR* c.-29G>A. GG and GT/TT refers to *FSHB* c.-211G>T.

[a] Additive model: WW vs WM vs MM, recessive model WW+WM vs MM, dominant model WW vs WM+MM.

[b] *P* for Effect (95% CI).

[c] $P < 0.001$.

[d] $P < 0.01$.

Unlike recent GWASs, we were able to integrate the effect of peripubertal BMI on pubertal timing (4, 5). BMI *z* score was strongly negatively associated with age at pubertal onset in both cohorts. The effect approximated a linear effect across the entire spectrum of normal BMI. Although the cohorts differed significantly in BMI *z* scores, the effect of BMI *z* score accounted for the same effect size per *z* score in both cohorts. Our results are in line with previous studies demonstrating a negative correlation of adolescent BMI with age at pubertal onset in normal-weight boys (6, 35, 36), potentially leveling off in overweight and even turning in obese boys, forming a curvilinear association (37). In our study, the effect of BMI on pubertal timing exceeded the influence of the investigated genetic variants. This highlights the necessity of correcting for BMI (if available) when analyzing factors possibly influencing pubertal timing. Thus, in the Chilean cohort, exhibiting a high mean BMI *z* score, the effect of *FSHB* c.-211G>T on pubertal timing became significant only when BMI was included in the model.

Given the complex and pulsatile secretion, peripubertal gonadotropin levels are highly variable and the clinical pubertal stage does not necessarily reflect the rise in gonadotropin levels accurately. Despite this variability, we were able to reproduce results of lower FSH level and an altered FSH/LH ratio comparable to previous large studies in adult men on *FSHB* c.-211G>T (13, 14, 38) underlining the variants' impact on gonadotropin levels.

Previous studies on the onset of puberty in boys and girls used clinical outcome measures (*e.g.*, age at menarche, age at voice break, self-reported Tanner stages) as proxies for pubertal onset. In this study, we used early testicular growth (*i.e.*, age at reaching a testicular volume ≥4 mL) as a clinical proxy for male pubertal onset. Boys with higher FSH action may reach the threshold testicular volume earlier than those with weaker FSH action, because FSH stimulates Sertoli cell proliferation and maturation, and drives testicular enlargement at early stages of pubertal development.

Our selective approach of assessing the effect of variation in specific genes affecting FSH action (a biological pathway essential for pubertal onset) minimizes the risk of false-positive associations. We have not assessed the effect of other polymorphisms.

In this longitudinal study of two genetically distinct clinical cohorts, we observed, to our knowledge, the largest effect of single genetic variants on age at male pubertal onset reported to date [*i.e.*, genetic variation altering FSH action (both *FSHB* c.-211G>T and *FSHR* c.-29A>G)].

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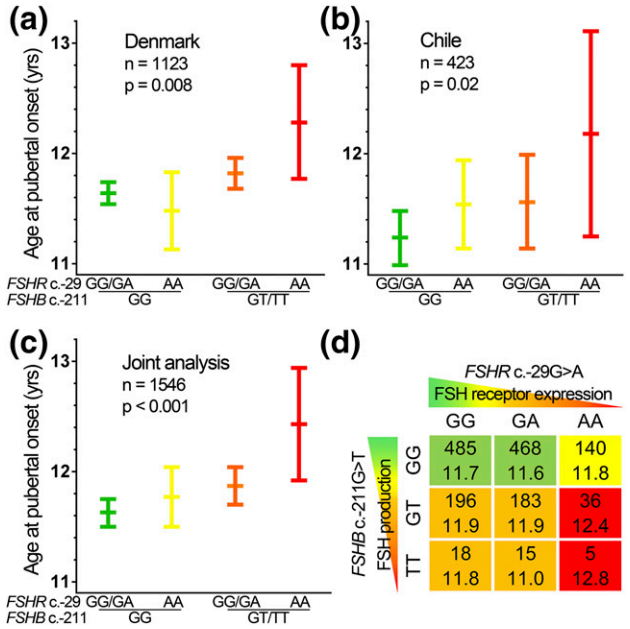

Figure 2. Age at pubertal onset of the combined genetic model of FSH action. Mean age at pubertal onset (95% CI) onset in (a) Danish boys (n = 1123), in (b) Chilean boys (n = 423) and (c) in joint analysis (n = 1546) stratified by *FSHB* c.-211G>T and *FSHR* c.-29G>A genotypes. Danish and Chilean data are corrected for BMI *z* score and the joint analysis is corrected for BMI *z* score and country of origin. (a–c) Colors of the bars correspond to the groups of (d) the combined genetic model of FSH action. (d) The color-coded boxes refer to (c) the joint analysis, with group size on the top line and mean age at pubertal onset on the bottom line of each box. (d) The bars fading from green to red indicate FSH production (10) and FSH receptor expression (11), respectively (from *FSHB* c.-211G>T/*FSHR* c.-29G>A wild-type homozygotes to minor-allele homozygotes).

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