Combined Effects of the Variants *FSHB* −211G>T and *FSHR* 2039A>G on Male Reproductive Parameters

Frank Tüttelmann, Maris Laan, Marina Grigorova, Margus Punab, Siim Sõber, and Jörg Gromoll

Institute of Human Genetics (F.T.) and Centre of Reproductive Medicine and Andrology (J.G.), University of Münster, 48149 Münster, Germany; and Institute of Molecular and Cell Biology (M.L., M.G., S.S.) and Andrology Unit (M.P.), Tartu University Clinics, University of Tartu, 51010 Tartu, Estonia

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Context: A polymorphism in the *FSHB* promoter (−211G>T, rs10835638) was shown to influence male serum FSH levels, whereas a polymorphism in the FSH receptor gene (*FSHR*; 2039A>G, rs6166) was previously shown to be associated with FSH levels in women only.

Objective: The objective of the study was to analyze the effects of both *FSHB* −211G>T and *FSHR* 2039A>G on male reproductive parameters.

Design and Setting: A total of 1213 German men attending an infertility clinic were genotyped by TaqMan assay.

Patients: Patients included male partners in infertile couples without known causes for male infertility.

Main Outcome Measures: An association analysis of single and combined single-nucleotide polymorphism genotypes with clinical parameters was performed.

Results: The *FSHB* −211G>T T-allele showed significant dosage effects for FSH (−0.51 U/liter per T-allele), LH (0.28 U/liter), and bitesticular volume (−3.2 ml). Statistical significance was enhanced severalfold after a meta-analysis comprising 3017 men. TT carriers were significantly more prevalent among men with lower sperm counts. The *FSHR* 2039A>G G-allele exhibited nonsignificant trends for associations with higher FSH and reduced testicular volumes. However, in the combined model, *FSHR* 2039A>G significantly modulated the more dominant effect of *FSHB* −211G>T on serum FSH and testicular volume among the T-allele carriers.

Conclusions: By analyzing both single-nucleotide polymorphisms for the first time, we convincingly show that indeed *FSHR* 2039A>G has an effect also in males. In the proposed model of the combined effects, *FSHB* −211G>T acts strongly on male reproductive parameters, whereas the *FSHR* 2039A>G effects were approximately 2–3 times smaller. Clinically this is of importance because oligozoospermic patients carrying unfavorable variants affecting FSH action may benefit from FSH treatment. **(*J Clin Endocrinol Metab* 97: 3639–3647, 2012)**

In males, FSH stimulates the proliferation of Sertoli cells during fetal, neonatal, and pubertal development through its receptor, whereas in adulthood, FSH is crucial for the maintenance of quantitative normal spermatogenesis (1, 2). Hence, the two genes, *FSHB* (encoding the specific β-subunit of mature FSH) and *FSHR* (encoding the FSH receptor), are prime candidates when studying spermatogenesis. To date, the specific function of FSH in spermatogenesis remains unclear because the few case reports of men with *FSHB*-inactivating mutations exhibited azoospermia, whereas inactivating mutations of the *FSHR* gene led to oligozoospermia (3). Nonetheless, to achieve a

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Abbreviations: FSHR, FSH receptor; SNP, single-nucleotide polymorphism; WHO, World Health Organization.

qualitatively and quantitatively normal spermatogenesis, a functioning FSH/FSH receptor (FSHR) interaction (in conjunction with testosterone) is necessary (4).

Single-nucleotide polymorphisms (SNPs) in both *FSHB* and *FSHR* have been studied separately for associations with reproductive parameters. Two common SNPs (c.919A>G, p.T307A, rs6165 and c.2039A>G, p.N680S, rs6166) are located in tight linkage in exon 10 of *FSHR* (5). The 2039A>G variant is regularly analyzed to characterize the exon 10 haplotype. Although no clear functional difference has been demonstrated for *FSHR* variants *in vitro* (6), in women the G-allele is associated with higher serum FSH concentrations, duration of the menstrual cycle, follicular growth dynamics, and response to ovarian stimulation (7, 8). In contrast, no association with male (in)fertility has been identified in seven original case-control studies summarized in three meta-analyses (9–11).

Recently a SNP in the *FSHB* gene (−211G>T, rs10835638) was found to be associated with lower serum FSH levels in cohorts of Baltic young men (12, 13). Concurrently an increased frequency of the T-allele in patients with oligozoospermia was shown (14), a finding that was confirmed in a later Italian study (15). The *FSHB* SNP is located 211 bp upstream from the transcription start site of the *FSHB* gene, and the G to T substitution leads to about 50% reduced transcriptional activity *in vitro* (16). The availability of FSH β-subunits has been found to be rate limiting for FSH assembly and secretion (17), and, fittingly, FSH serum levels were found to be significantly decreased in homozygous carriers of the *FSHB* −211 T-allele with heterozygotes having intermediate levels (12–15).

Because no study addressing joint effects of both *FSHB* −211G>T and *FSHR* 2039A>G has been conducted so far, we analyzed a large sample of 1213 German men who attended our infertility clinic to elucidate the impact of the two SNP on the reproductive parameters.

Subjects and Methods

Study population

All men were of Caucasian ethnicity and German origin according to self-report and attended the Department of Clinical Andrology, Centre of Reproductive Medicine and Andrology, University Clinic Münster, a tertiary-referral center for infertility. The primary study group was retrospectively selected from the patient clientele between 2000 and 2010 using the Androbase database (18). This group comprised 1213 nonazoospermic patients without known clinical (*e.g.* oncological disease including testicular tumors, maldescended testes, varicocele, infections) or genetic (karyotype anomalies, Y-chromosomal deletions) causes of infertility. Approximately half of the men had sperm concentrations greater than 20 million per milliliter (n = 615) and 12.2% (n = 148) had spontaneously fathered a child in their current or a former relationship. We therefore refrain from using the term "idiopathic infertile patients," but instead we use the term "men with couple infertility". Seven hundred fifty-five patients having one or more of the aforementioned clinical and/or genetic causal factors were consecutively recruited between January and October 2011. All participants underwent a complete physical examination including ultrasonographic analysis of their scrotal contents. Testicular volume was calculated using the ellipsoid method and summed as bitesticular volume.

Ethics statement

All participants gave informed written consent for evaluation of their clinical data and genetic analysis of their donated DNA samples according to protocols approved by the Ethics Committee of the Medical Faculty in Münster.

Hormone and semen analyses

A venous blood sample was drawn from the cubital vein between 0800 and 1330 h. Serum concentrations of FSH and LH were determined by immunofluorometric assays (Autodelfia; Perkin-Elmer, Freiburg, Germany) and serum testosterone by a commercial ELISA (DRG AURICA ELISA testosterone kit; DRG Instruments, Marburg, Germany). Intra- and interassay coefficients of variation were less than 5% and less than 10%, respectively.

Ejaculates were obtained by masturbation and all semen parameters were determined in accordance with World Health Organization (WHO) criteria (19, 20). Sperm concentration was analyzed with Neubauer-improved chambers, and motility was assessed on a heated microscope stage. Slides for morphology assessment were stained with the modified Papanicolaou method and examined with phase-contrast optics. Progressive motility comprises WHO 1999 categories a and b. Total sperm count was calculated by multiplying semen volume by sperm concentration.

All hormone assays and semen analysis were under constant internal and external quality control.

Genotyping

Genomic DNA was extracted from EDTA-preserved blood using the FlexiGene DNA extraction kit (QIAGEN, Düsseldorf, Germany). The polymorphisms of the *FSHB* (rs10835638) and *FSHR* (rs6166) genes were analyzed by PCR and allelic discrimination assay on the ABI PRISM 7000 detection system (Applied Biosystems, Darmstadt, Germany). Each PCR contained 5 μl genomic DNA, 1 μl Assaymix, 10 μl TaqMan genotyping master mix, and 4 μl A. bidest. The TaqMan SNP genotyping assay C_27829553_10 for *FSHB* and C_2676874_10 for *FSHR* were used. The PCR cycles were as follows: 1) 50 C, 2 min (one cycle) for probe binding, 2) denaturation at 95 C 15 min (one cycle), and 3) 95 C for 15 sec and 60 C for 1 min (35 cycles). Allelic discrimination assay took 1 min at 60 C. *FSHR* 2039A>G was only genotyped in the retrospectively selected 1213 men.

Statistical analysis

All analyses were carried out with Stata/SE software (StataCorp LP, version 9.1; College Station, TX) partly using specific genetic subroutines (http://www.biostat-resources.com): HWSNP for departure from Hardy-Weinberg equilibrium and GENCC to test differences in allele and genotype fre-

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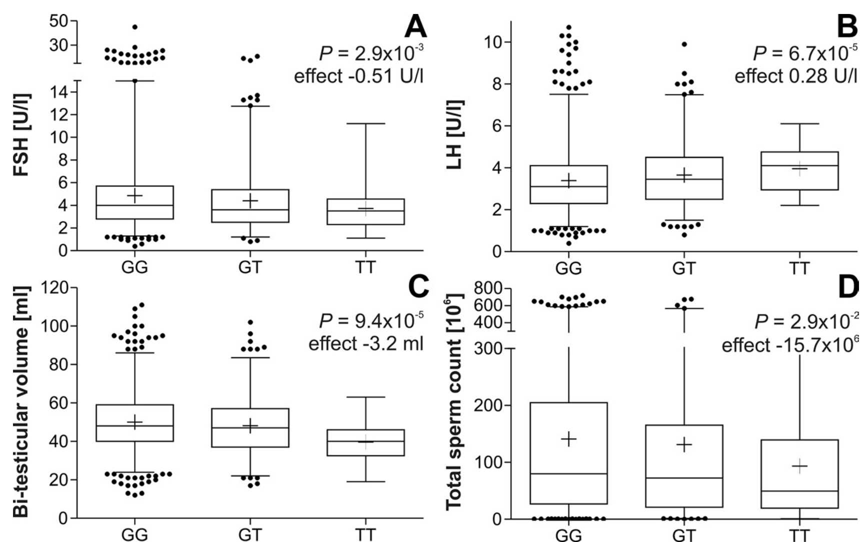

FIG. 1. Box-and-whisker plots of FSH (A), LH (B), bitesticular volume (C), and total sperm count (D) according to *FSHB* −211G>T genotype in the German study group without causal factors (n = 1213). *Boxes* contain 50% of the distribution, and whiskers extend to 5–95%; *lines in boxes* represent the median and *crosses* the mean. *P*-values calculated by linear regression across genotypes with the covariate age and additionally abstinence time for sperm count.

quencies both calculating Fisher's exact and χ^2 statistics. QTLSNP (quantitative trait loci SNP) uses linear regression to compare the equality of means of clinical parameters across genotypes. The additive model assumes a codominant effect of alleles in which the heterozygotes should exhibit intermediate levels; regressions are calculated over, for example, GG *vs.* GT *vs.* TT. In the recessive model, strong effects are expected only in minor allele homozygotes, and pooled wild-type and heterozygotes are compared with homozygotes (*e.g.* GG+GT *vs.* TT). Because hormone and semen parameters were not normally distributed, calculations were carried out on log-transformed data. Age was introduced as covariate for all parameters and additionally abstinence time for semen parameters. The associations between *FSHB* −211G>T, and clinical parameters were recalculated for the previously published Baltic studies under the same settings as the Münster study group to accomplish the best possible homogeneity, and then meta-analysis was performed with the meta package (http://cran.r-project.org/webpackages/meta/index.html) for the statistical package R (http://www.r-project.org) using inverse variance method under the fixed-effects model. $P < 0.05$ was primarily considered statistically significant. Additionally, a more conservative $P = 3.1 \times 10^{-3}$ was applied after Bonferroni correction for two genetic models and eight independent parameters [$P = 0.05/(2 \times 8)$].

Results

By retrospectively selecting 1213 men with couple infertility from our center's registry and adding 755 consecutively recruited patients, we present the largest unique study groups analyzed for *FSHB* −211G>T and *FSHR* 2039A>G so far. Clinical characteristics of the study populations are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org. The two groups were significantly different in all hormone and semen parameters ($P < 0.001$) except duration of abstinence ($P = 0.42$). The allele frequencies and the genotype distribution of *FSHB* −211G>T were in Hardy-Weinberg equilibrium in both groups ($P = 0.36$ and $P = 0.49$, respectively) and likewise for *FSHR* 2039A>G in the men without causal factors ($P = 0.67$).

Effects of *FSHB* −211G>T

In the men without causal factors for infertility, the quantitative trait analysis using an additive genetic model revealed significant negative associations for FSH (T-allele effect, *i.e.* β-coefficient of regression −0.51 U/liter, $P = 2.9 \times 10^{-3}$), FSH to LH ratio (−0.24 U/liter, $P = 7.8 \times 10^{-11}$), and testicular volume (−3.2 ml, $P = 9.4 \times 10^{-5}$) as well as sperm concentration (-6.7×10^6/ml, $P = 4.8 \times 10^{-2}$) and count (-15.7×10^6, $P = 2.9 \times 10^{-2}$) (Fig. 1 and Table 1). In contrast, LH levels increased with the number of T-alleles (0.28 U/liter, $P = 6.7 \times 10^{-5}$). Men homozygous for the T-allele had 24% lower FSH, 18% higher LH, 21% lower testicular volumes, 36% lower sperm concentrations, and 34% lower total sperm counts compared with GG homozygotes. All of these associations, except sperm concentration/count, remained significant after conservative Bonferroni correction ($P = 3.1 \times 10^{-3}$). Overall, similar associations were found in men with causal factors for infertility (Supplemental Table 2). However, the sizes of the effects in these patients were less pronounced (lower effect sizes), and associations either did not reach statistical significance or had lower significance levels (*P* values).

Meta-analysis of *FSHB* −211G>T including previous studies in Baltic populations

To further substantiate our findings on the *FSHB* −211G>T SNP and increase the statistical power, we conducted a meta-analysis including all men without causal factors in the current study (n = 1213), the previously published data on Estonian idiopathic infertility patients (n = 750) (14) and the Baltic young men cohort (n = 1054) (13). In the meta-analysis (Table 2), FSH and FSH to LH ratio were unambiguously associated with *FSHB* −211G>T genotype in all study groups and reached combined *P* values for the additive model of 1.5×10^{-8} and 2.2×10^{-16}. Testicular volume showed a trend toward

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TABLE 1. Clinical parameters of the German study group without causal factors (n = 1213) stratified by *FSHB* −211G>T genotype

FSHB −211G>T	G/G (n = 852)	G/T (n = 324)	T/T (n = 37)	Additive model[a] *P* value	Additive model[a] T-allele effect	Recessive model[a] *P* value	Recessive model[a] TT homozygosity effect
FSH (U/liter)	4.9 ± 3.7 4.0 (1.7–10.7)	4.4 ± 2.9 3.6 (1.6–10.3)	3.7 ± 1.9 3.5 (1.5–7.2)	**2.9×10^{-3}**	**−0.51 (0.19)**	6.2×10^{-2}	−1.01 (0.58)
LH (U/liter)	3.4 ± 1.6 3.1 (1.5–6.4)	3.7 ± 1.6 3.5 (1.7–6.6)	4.0 ± 1.1 4.1 (2.3–6)	**6.7×10^{-5}**	**0.28 (0.08)**	**9.5×10^{-3}**	**0.49 (0.26)**
FSH/LH	1.5 ± 0.9 1.3 (0.6–3.1)	1.3 ± 0.7 1.1 (0.5–2.5)	1.0 ± 0.6 0.9 (0.3–2.7)	**7.8×10^{-11}**	**−0.24 (0.04)**	**3.5×10^{-5}**	**-0.45 (0.14)**
Testosterone (nmol/liter)	15.5 ± 5.5 14.8 (8.4–25.8)	15.9 ± 5.5 15.5 (8.8–24.4)	15.4 ± 5.6 14.5 (7.7–27.4)	2.3×10^{-1}	0.31 (0.29)	7.7×10^{-1}	−0.13 (0.91)
Bitesticular volume (ml)	50.0 ± 15.4 48 (27–77)	48.1 ± 14.9 47 (25–74)	39.7 ± 10.7 40 (20–63)	**9.4×10^{-5}**	**−3.20 (0.83)**	**7.3×10^{-5}**	**−9.84 (2.53)**
Sperm concentration (10^6/ml)	40.0 ± 47.6 21.0 (0.7–140.7)	34.3 ± 41.3 18.8 (0.8–136.8)	25.5 ± 29.8 14 (0.8–102.7)	**4.8×10^{-2}**	**−6.66 (2.48)**	2.4×10^{-1}	−13.19 (7.62)
Semen volume (ml)	3.9 ± 1.6 3.6 (1.7–7.0)	4.0 ± 1.9 3.5 (1.5–8.1)	3.6 ± 1.5 3.5 (1.5–6.6)	8.4×10^{-1}	0.02 (0.09)	3.5×10^{-1}	−0.26 (0.28)
Total sperm count (10^6)	141.0 ± 159.5 80.0 (2.4–474.6)	131.2 ± 181.2 72.3 (2.5–447.3)	93.6 ± 108.7 49.6 (1.4–351.9)	**2.9×10^{-2}**	**−15.69 (8.92)**	1.3×10^{-1}	−44.45 (27.40)
a+b motility (%)	45.5 ± 13.2 48 (18–63)	44.3 ± 13.9 47 (13–62)	45.0 ± 11.8 48 (20–64)	1.9×10^{-1}	−0.93 (0.71)	8.5×10^{-1}	−0.42 (2.2)
Normal morphology (%)	12.1 ± 7.9 10 (2–28)	11.8 ± 7.0 10 (3–26)	11.6 ± 6.5 11 (3–23)	4.7×10^{-1}	−0.30 (0.42)	7.2×10^{-1}	−0.46 (1.27)

Data are presented as mean ± SD and median (fifth to 95th percentile). Genotype-trait associations are calculated by linear regression across genotypes with the covariate age for all and additionally abstinence time for semen parameters. Significant associations are marked in *bold* ($P < 5.0 \times 10^{-2}$) or *bold+underlined* ($P < 3.1 \times 10^{-3}$) and *underlined* for trends ($P < 1.0 \times 10^{-1}$).

[a] The additive model assumes a codominant effect of alleles in which the heterozygotes should generally exhibit intermediate levels; regressions are calculated over GG *vs.* GT *vs.* TT. In the recessive model, strong effects are expected only in minor allele homozygotes, and therefore, pooled wild-type and heterozygotes are compared with homozygotes (GG+GT *vs.* TT). The β-coefficients of regression provide T-allele and TT homozygosity effects, respectively, and are presented with SE in *parentheses*.

formal significance in the Estonian patients and was highly significantly associated with the *FSHB* −211G>T genotype in all other groups reflected by a combined *P* value of 2.0×10^{-7} in the recessive model. In contrast, sperm concentration and total sperm count were significantly associated only with *FSHB* −211G>T in the German men without causal factors.

Effects of *FSHR* 2039A>G

The study group of men without causal factors (n = 1213) was also genotyped for *FSHR* 2039A>G. In contrast to *FSHB* −211G>T, no significant associations between *FSHR* 2039A>G and any of the clinical parameters analyzed were identified (Supplemental Table 3). Nonsignificant gradients of increasing FSH (4.7/4.6/4.8 U/liter,

TABLE 2. Meta-analysis of *FSHB* −211G>T in all men without causal factors

Parameter	Genetic model	German men with couple infertility (n = 1213) *P* value	German Effect[a]	Estonian men with couple infertility (n = 750) *P* value	Estonian Effect[a]	Baltic young men cohort (n = 1054) *P* value	Baltic Effect[a]	Joint meta-analysis (n = 3017) *P* value	Joint Effect[a]
FSH (U/liter)	Additive	**2.9×10^{-3}**	**−0.51 (0.19)**	**1.4×10^{-7}**	**−2.25 (0.59)**	**4.3×10^{-7}**	**−0.48 (0.11)**	**1.5×10^{-8}**	**−0.53 (0.09)**
	Recessive	6.2×10^{-2}	−1.01 (0.58)	**6.7×10^{-4}**	**−3.96 (1.93)**	6.5×10^{-2}	−0.66 (0.45)	**1.0×10^{-2}**	**−0.9 (0.35)**
LH (U/liter)	Additive	**6.7×10^{-5}**	**0.28 (0.08)**	7.8×10^{-1}	−0.14 (0.19)	3.0×10^{-1}	0.13 (0.11)	**1.9×10^{-3}**	**0.19 (0.06)**
	Recessive	**9.5×10^{-3}**	**0.49 (0.26)**	7.6×10^{-1}	0.13 (0.62)	**3.1×10^{-2}**	**1.09 (0.45)**	**6.1×10^{-3}**	**0.58 (0.21)**
FSH/LH	Additive	**7.8×10^{-11}**	**−0.24 (0.04)**	**9.5×10^{-10}**	**−0.41 (0.09)**	**7.4×10^{-9}**	**−0.14 (0.03)**	**2.2×10^{-16}**	**−0.19 (0.02)**
	Recessive	**3.5×10^{-5}**	**−0.45 (0.14)**	**1.4×10^{-5}**	**−0.93 (0.28)**	**3.9×10^{-4}**	**−0.31 (0.12)**	**1.1×10^{-6}**	**−0.42 (0.09)**
Testosterone (nmol/liter)	Additive	2.3×10^{-1}	0.31 (0.29)	2.2×10^{-1}	0.54 (0.48)	**3.1×10^{-3}**	**−1.67 (0.60)**	7.5×10^{-1}	0.07 (0.23)
	Recessive	7.7×10^{-1}	−0.13 (0.91)	6.6×10^{-1}	−1.05 (1.53)	3.0×10^{-1}	−2.59 (2.44)	4.4×10^{-1}	−0.58 (0.74)
Bitesticular volume (ml)	Additive	**9.4×10^{-5}**	**−3.20 (0.83)**	2.3×10^{-1}	−1.31 (0.77)	**2.7×10^{-2}**	**−1.28 (0.67)**	**2.8×10^{-5}**	**−1.81 (0.43)**
	Recessive	**7.3×10^{-5}**	**−9.84 (2.53)**	9.0×10^{-2}	−5.13 (2.49)	**1.6×10^{-4}**	**−8.4 (2.72)**	**2.0×10^{-7}**	**−7.73 (1.49)**
Sperm concentration (10^6/ml)	Additive	**4.8×10^{-2}**	**−6.66 (2.48)**	1.9×10^{-1}	0.52 (0.44)	4.2×10^{-1}	−5.1 (4.81)	5.5×10^{-1}	0.26 (0.43)
	Recessive	2.4×10^{-1}	−13.19 (7.62)	7.5×10^{-1}	−0.17 (1.43)	1.4×10^{-1}	−25.77 (19.48)	6.0×10^{-1}	−0.74 (1.4)
Semen volume (ml)	Additive	8.4×10^{-1}	0.02 (0.09)	6.6×10^{-1}	0.06 (0.13)	7.1×10^{-1}	0.04 (0.10)	5.5×10^{-1}	0.04 (0.06)
	Recessive	3.5×10^{-1}	−0.26 (0.28)	5.7×10^{-1}	0.24 (0.42)	2.1×10^{-1}	0.53 (0.42)	8.3×10^{-1}	0.04 (0.2)
Total sperm count (10^6)	Additive	**2.9×10^{-2}**	**−15.69 (8.92)**	1.4×10^{-1}	2.18 (2.27)	6.4×10^{-1}	−15.01 (17.69)	7.0×10^{-1}	0.85 (2.18)
	Recessive	1.3×10^{-1}	−44.45 (27.40)	8.4×10^{-1}	−2.13 (7.33)	3.8×10^{-1}	−31.39 (71.63)	4.6×10^{-1}	−5.21 (7.05)

Significant associations are marked in *bold* ($P < 5.0 \times 10^{-2}$) or *bold+underlined* ($P < 3.1 \times 10^{-3}$) and *underlined* for trends ($P < 1.0 \times 10^{-1}$).

[a] Effect = either T-allele or TT homozygosity effect (β-coefficient of regression), depending on the genetic model (additive or recessive) with SE in *parentheses*.

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TABLE 3. Allele and genotype frequencies of *FSHB* −211G>T and *FSHR* 2039A>G in all men available stratified into tertiles based on total sperm count (percentage; number in *parentheses*)[a]

	German (n = 1213) and Estonian (n = 744) men with couple infertility and Baltic young men cohort (n = 1032) (n = 2989 total)		
***FSHB* −211G>T Total sperm count**	**≤38.9 million (n = 998)**	**38.9–162.8 million (n = 995)**	**>162.8 million (n = 996)**
Allele frequencies			
G	84.4% (1684)	83.7% (1665)	87.4% (1742)
T	15.6% (312)	16.3% (325)	12.6% (250)
		$P = 1.7 \times 10^{-3}$	
Genotype frequencies			
G/G	71.9% (718)	69.7% (694)	76.3% (760)
G/T	24.8% (248)	27.8% (277)	22.3% (222)
T/T	3.2% (32)	2.4% (24)	1.4% (14)
		$P = 2.8 \times 10^{-3}$	
	German men with couple infertility (n = 1213)		
***FSHR* 2039A>G Total sperm count**	**≤39.2 million (n = 405)**	**39.2–144.0 million (n = 404)**	**>144.0 million (n = 404)**
Allele frequencies			
A	53.6% (434)	55.2% (446)	52% (420)
G	46.4% (376)	44.8% (362)	48% (388)
		$P = 4.3 \times 10^{-1}$	
Genotype frequencies			
A/A	28.4% (115)	31.9% (129)	26.7% (108)
A/G	50.4% (204)	46.5% (188)	50.5% (204)
G/G	21.2% (86)	21.5% (87)	22.8% (92)
		$P = 5.5 \times 10^{-1}$	

First tertile corresponds to the WHO 2010 reference range of oligozoospermia.

[a] *P* value of χ^2 test for allele and genotype frequencies of men with low, medium, or high sperm counts.

$P = 4.8 \times 10^{-1}$) as well as LH (3.4/3.5/3.6 U/liter, $P = 5.8 \times 10^{-2}$) and decreasing testicular volume (50.1/49.1/48.3 ml, $P = 1.6 \times 10^{-1}$) across the *FSHR* 2039A>G genotype subgroups (AA/AG/GG) were observed.

Allele and genotype frequencies

Although the marker trait analyses provide biologically important information, clinically relevant results can be obtained from case-control analyses. We took advantage of the large available dataset of German/Baltic men with sperm counts (n = 2989). The subjects were stratified based on their total sperm counts into tertiles (>162.8 million, n = 996; 38.9–162.8 million, n = 995; < 38.9 million, n = 998; Table 3) with the lowest tertile corresponding to the current WHO threshold for oligozoospermia (20). The analysis revealed the lowest prevalence of T-allele carriers among men with the highest sperm counts ($P = 1.7 \times 10^{-3}$). The frequency of TT carriers among men with sperm counts below 39 million (3.2%) was found to be more than twice that of those with high sperm counts (1.4%). When men were stratified based on the previously applied WHO threshold (19) of 20 million /ml for sperm concentration (Supplemental Table 4), the calculated odds ratio for TT carriers being oligozoospermic was 2.1 (95% confidence interval 1.3–3.7). In contrast, neither allele frequencies nor the genotype distribution of *FSHR* 2039A>G differed in the 1213 German men without causal factors (Table 3).

Combined analysis of SNPs in *FSHB* and *FSHR*

Finally, the men without causal factors were stratified into nine subgroups according to their combined *FSHB* −211G>T and *FSHR* 2039A>G genotypes (Table 4). No significant differences were found for any parameter among the *FSHB* −211 GG carriers. However, increasing FSH and LH levels as well as decreasing testicular volumes along the *FSHR* 2039A>G genotypes were observed among the *FSHB* −211 GT and TT carriers (Table 4). Even though a large number of men was analyzed in total, the number of individuals especially among the substratified TT carriers was low, and the analyses did not withstand the most stringent Bonferroni correction (all $P > 3.1 \times 10^{-3}$). Based on available *in vivo* and *in vitro* studies, we propose a model of *FSHB* −211G>T and *FSHR* 2039A>G (Fig. 2A). Decreasing transcriptional activity (*FSHB* −211G>T, stronger effect) leads to lower serum FSH, and decreasing receptor sensitivity (*FSHR* 2039A>G, weaker effect) leads to higher FSH, whereas both lead to a decrease of testicular

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TABLE 4. Clinical parameters of the German study group without causal factors (n = 1213) stratified by *FSHB* −211G>T and *FSHR* 2039A>G genotype

	G/G			G/T			T/T		
***FSHB* −211G>T** / ***FSHR* 2039A>G**	**A/A (n = 261)**	**A/G (n = 402)**	**G/G (n = 189)**	**A/A (n = 85)**	**A/G (n = 173)**	**G/G (n = 66)**	**A/A (n = 6)**	**A/G (n = 21)**	**G/G (n = 10)**
FSH (U/liter)	4.9 ± 4.3	4.9 ± 3.5	4.8 ± 3.3	4.4 ± 2.6	4.2 ± 2.9	4.8 ± 3.1	**2.6 ± 0.4**	**3.6 ± 1.6**	**4.7 ± 2.7**
	3.8 (1.7–11.3)	4.0 (1.8–11.2)	4.1 (1.5–10.3)	3.6 (1.7–10.5)	3.3 (1.3–10.7)	3.9 (1.6–9.6)	**2.6 (1.9-3.0)**	**3.7 (1.1-6.7)**	**3.9 (1.6-11.2)**
LH (U/liter)	3.4 ± 1.6	3.4 ± 1.6	3.4 ± 1.4	**3.4 ± 1.4**	**3.7 ± 1.7**	**4.0 ± 1.5**	3.7 ± 1.0	3.9 ± 1.1	4.2 ± 1.2
	3 (1.5–6.3)	3.1 (1.5–6.7)	3.2 (1.6–5.9)	**3.2 (1.5-5.8)**	**3.4 (1.7-7)**	**3.8 (1.8-7.1)**	3.5 (2.5–5.2)	4.1 (2.2–6.0)	4.1 (2.4–6.1)
FSH/LH	1.5 ± 0.9	1.5 ± 0.8	1.5 ± 0.9	1.4 ± 0.9	1.2 ± 0.6	1.3 ± 0.8	0.7 ± 0.2	1.0 ± 0.6	1.1 ± 0.7
	1.3 (0.6–3.1)	1.3 (0.6–3.0)	1.3 (0.5–3.2)	1.2 (0.6–3.5)	1.1 (0.5–2.2)	1.1 (0.3–3.1)	0.8 (0.5–0.9)	1.0 (0.3–2.6)	1.0 (0.6–2.9)
Testosterone (nmol/liter)	15.8 ± 5.9	15.3 ± 5.2	15.3 ± 5.5	15.8 ± 4.9	16.2 ± 6.0	15.2 ± 4.7	15.3 ± 6.4	15.3 ± 6.2	15.8 ± 3.8
	14.8 (8–27.1)	14.9 (8.6–24.1)	14.2 (8–26.7)	15.6 (8.8–24.2)	15.6 (8.8–25.2)	14.9 (7.7–24.3)	13.9 (8.8–27.3)	14.4 (4.9–27.7)	15.4 (9.7–21.6)
Bitesticular volume (ml)	49.9 ± 14.9	50 ± 15.7	50.2 ± 15.7	**51.1 ± 16.1**	**47.7 ± 14.6**	**45.4 ± 13.9**	43.3 ± 5.2	41.9 ± 11.8	32.8 ± 7.7
	48 (28–79)	49 (26–75)	47 (29–82)	**51 (30-80)**	**48 (25-74)**	**44 (25-75)**	42 (39–53)	41 (19–63)	31 (23–46)
Sperm concentration (10^6/ml)	34.2 ± 39.7	41.6 ± 51	44.4 ± 49.3	36.1 ± 40.1	35.5 ± 43.3	28.8 ± 37.5	23.4 ± 26.1	31.1 ± 36	14.9 ± 9.7
	19.6 (0.6–114.5)	21.9 (0.6–143.9)	25.5 (1.3–153.8)	20.5 (0.6–135.9)	19.5 (0.9–141.3)	16 (0.7–136.7)	16.8 (0.5–74)	17.3 (0.9–108.3)	12.8 (1.8–34.8)
Semen volume (ml)	3.8 ± 1.6	3.9 ± 1.7	3.9 ± 1.6	4.0 ± 1.8	3.9 ± 1.9	4.1 ± 2.0	4.2 ± 1.5	3.4 ± 1.3	3.6 ± 2.1
	3.6 (1.5–7.3)	3.6 (1.8–7)	3.6 (1.7–6.8)	3.5 (1.8–7.4)	3.5 (1.4–8.2)	3.8 (1.7–8.2)	4.3 (1.7–5.8)	3.4 (1.6–6.5)	3.5 (0.9–7.4)
Total sperm count (10^6)	124.2 ± 134.6	144.4 ± 168.3	156.7 ± 170.5	142.3 ± 178.5	134.2 ± 199.8	109 ± 125.1	110.3 ± 121.8	103.6 ± 124.1	62.4 ± 57.5
	70.0 (1.8–416.1)	82.6 (2.7–491.2)	89.9 (3.6–498.8)	73.1 (1.9–532.7)	65.9 (2.3–446.0)	68.9 (3.5–375.7)	78.4 (0.9–340.4)	42.6 (1.7–438.9)	40.7 (3.2–161.3)
a+b motility (%)	44.7 ± 12.6	45.3 ± 13.8	47.0 ± 12.6	45.9 ± 13.5	43.5 ± 14.2	44.4 ± 13.9	43.3 ± 13.2	44.3 ± 11.4	47.3 ± 12.6
	47 (18–61)	48 (17–64)	50 (23–65)	50 (15–64)	46 (12–62)	46 (14–62)	45 (27–62)	47 (20–62)	49.5 (20–69)
Normal morphology (%)	12.1 ± 8.2	11.9 ± 7.7	12.7 ± 7.9	12.7 ± 7.3	11.7 ± 7	10.8 ± 6.6	11.3 ± 7.3	11.9 ± 6.5	11.1 ± 6.6
	10 (2–30)	10 (2–27)	11 (3–31)	11 (3–27)	11 (3–26)	9 (2–25)	11.5 (3–23)	11 (2–22)	10 (4–26)

Data are presented as mean ± SD and median (fifth to 95th percentile). Genotype-trait associations are calculated by linear regression across genotypes with the covariate age for all and additionally abstinence time for semen parameters. Significant associations marked in **bold** ($P < 5.0 \times 10^{-2}$) or underlined for trends ($P < 1.0 \times 10^{-1}$).

volume. Fitting data for both parameters is presented in Fig. 2, B and C. For *FSHB* −211 T-allele carriers, the gradient of FSH over the combined genotype groups was marginally significant (*FSHR* 2039A>G AA, 2.6 U/liter; AG, 3.6 U/liter; GG, 4.7 U/liter; $P = 4.3 \times 10^{-2}$), whereas the decrease in testicular volumes was highly significant (*FSHR* 2039A>G AA, 43.3 ml; AG, 41.9 ml; GG, 32.8 ml; $P = 1.7 \times 10^{-5}$). We additionally performed a multiple linear regression analysis on *FSHB* −211 T-allele carriers, including both SNPs, which revealed that the effect of the *FSHB* −211 T-allele was about 3 times that of the *FSHR* 2039 G-allele for FSH (−0.71 *vs.* 0.23 U/liter) and more than twice as high for testicular volume (−7.9 *vs.* −3.2 ml) (data not shown). Fitting to the model, carriers of *FSHB* TT and *FSHR* AA had the lowest FSH levels, whereas carriers of the TT/GG combination had the lowest testicular volumes.

Discussion

Many polymorphisms have been studied as possible risk factors for male infertility but have either not been replicated or their clinical value remains debatable because consequences after testing are lacking (9). A similar situation was true for the *FSHR* exon 10 SNP 2039A>G. Whereas clear phenotypic effects in women have been demonstrated (7–11), associations with male (in)fertility are ambiguous at best (9–11). In contrast, three Baltic and an Italian study consecutively provided evidence of an influence of the newly studied *FSHB* promoter SNP −211G>T on serum FSH and other reproductive parameters (12–15). We analyzed a group of 1213 well-characterized men without, and 755 men with, causal factors seeking consultation for couple infertility. Exceptionally large groups when compared with many of the other studies in male fertility research but ones that are necessary to pinpoint what can be modest SNP effects. For the first time, we show an influence of *FSHR* 2039A>G on FSH levels and testicular volume secondary to the stronger effects of *FSHB* −211 G/T. The combinatory effects of the two SNPs of the hormone and its receptor make *FSHB* −211G>T/*FSHR* 2039A>G an unparalleled example in reproductive endocrinology.

The *FSHB* promoter's SNP reduces transcription of *FSHB in vitro* (16), and a close correlation between the availability of FSH β-subunits with FSH assembly and secretion has been postulated (17). Fittingly, we verified a close association between *FSHB* −211G>T and serum FSH levels in German men (24% lower in TT homozygotes compared with GG homozygotes). In contrast, our study showed LH serum levels that were 18% higher in TT carriers, findings that are comparable with the previous observations in the Baltic studies. Whereas testosterone levels were comparable between all genotype groups in our German men and Estonian men with couple infertility (12), lower testosterone levels were seen in Baltic young men either hetero- or homozygous for the T-allele (13). A higher hypothalamic drive on the pituitary to compensate for the lower amount of FSH β-subunits may explain the observed higher LH levels in TT subjects. The decreased

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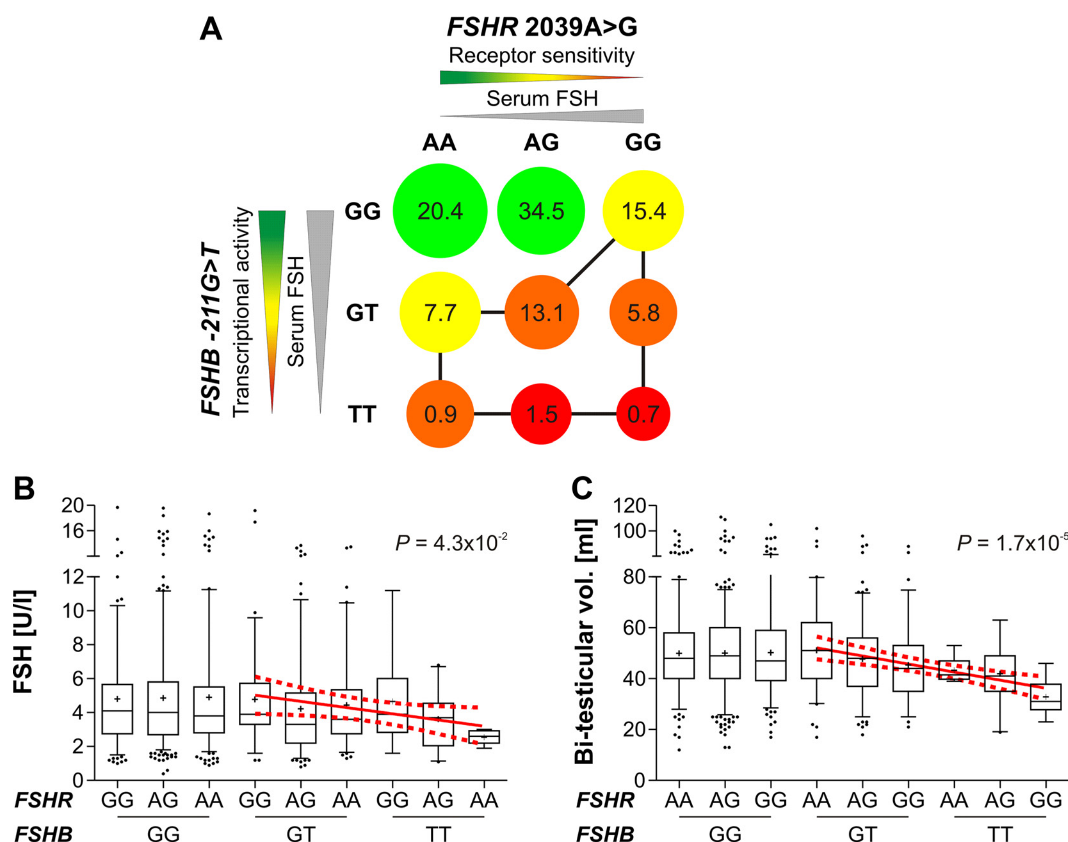

FIG. 2. Proposed model of *FSHB* −211G>T and *FSHR* 2039A>G effects. A, Decreasing transcriptional activity (*FSHB* −211G>T, stronger effect) and receptor sensitivity (*FSHR* 2039A>G, weaker effect) both lead to a decrease of testicular volume depicted by circle diameter. *Colors* represent genotypes with better (*green*) and worse (*red*) putative impact on reproductive fitness. *Numbers in circles* reflect percentage of carriers of combined genotypes in the studied population. The lowest testicular volumes are predicted for TT/GG carriers. Those men with the least favorable genotype combinations are connected with the *black line*. Observed FSH (B) and bitesticular volume (C) in the German study group without causal factors (n = 1213) including regressions over the means of *FSHB* −211G>T T-allele carriers (last six genotype combinations) depicted by *red line* with 95% confidence interval and respective *P* values. Note the different order of *FSHR* genotypes between B and C.

testosterone levels might be due to the reduced Sertoli cell numbers (see discussion below) which might in turn negatively influence Leydig cell function. This putative interaction is supported by case reports on three men homozygous for inactivating mutations in *FSHB* who were found to have delayed puberty, presumably caused by disturbed Leydig cell function (21). All similarly affected women have also been found to have elevated LH levels (3).

Beyond the *FSHB* −211G>T genotype's association with serum FSH, we found it to also be related to decreased testicular volume (21% lower in TT homozygotes compared with GG homozygotes). However, the ratio of total sperm count to testicular volume, *i.e.* sperm production per volume of testicular tissue as a marker of spermatogenic efficiency, was found to be similar in all genotype groups (data not shown), indicating that it is not influenced by carrier status of the SNP alleles. Because each individual Sertoli cell is in contact with a defined number of spermatozoa, the number of Sertoli cells is the major determinant of adult spermatogenic output and testicular size (22, 23). In conjunction, these observations hint that a fetal, neonatal, and/or pubertal effect induced by lower FSH levels may be responsible for the observed smaller testes.

Sperm concentration/count were found to be associated with *FSHB* −211G>T in the German men with TT carriers having 34% lower total sperm counts, findings similar to three of the four previous studies (12–15). Because these associations fit the observed effects on testicular size, our data support the suggestion that a connection between *FSHB* −211G>T and spermatogenic output exists. Most likely because sperm concentration and count are known to exhibit high intraindividual variability, the examination of even larger numbers of subjects may be needed to reveal significant underlying effects. When considering the case-control analyses, a clear relationship between carriership of *FSHB* −211G>T and lower sperm counts was established for the first time with TT carriers having an odds ratio of 2.1 to belong to the oligozoospermic group. Altogether our analyses demonstrate that *FSHB* −211G>T is associated with variations in FSH serum levels, testicular size, and spermatogenesis, is a risk factor for oligozoospermia, and consequently adversely influences male fertility.

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Our findings underline the importance of selecting clinically well-characterized and homogenous (idiopathic) groups to eliminate as many confounding factors as possible and thus increase the chances of identifying significant genotype-trait associations. However, from the clinical point of view, an analysis of nonidiopathic patients, like our additional study group of men with causal factors for infertility, is also of importance because these comprise the majority of about 70% of patients seeking consultation (24, 25). Even though the relationships with *FSHB* −211G>T were less pronounced in this group of patients, the corresponding effects point in the same directions. Because the influence of this SNP seems to be present also in nonidiopathic infertile patients, it can be surmised that it acts independently (on top) of the main cause of spermatogenic failure.

Since the first study detected an influence of *FSHR* 2039A>G on FSH levels and reproductive parameters in women (7, 8), it has been intriguing that thus far no clear effects have been established in men. In line with previous studies and meta-analyses (9–11), our single analyses of 2039A>G did not reveal any significant associations with the hormone or semen parameters in the German men with couple infertility. However, in this large data set, trends of higher FSH and lower testicular volume were observed in G-allele carriers, which parallel some previous observations (26, 27). By combining the analyses of *FSHB* −211G>T and *FSHR* 2039A>G for the first time, we clearly show that 2039A>G does have effects also on male reproductive function. Based on our findings, we propose an explanatory model (Fig. 2), showing how combinations of the two SNP minor alleles may be responsible for the worst phenotype of lowest testicular volumes. In contrast, the higher sensitivity of the receptor in *FSHR* 2039A>G AA homozygotes may partly compensate for the lower serum FSH, which are caused by the reduced transcriptional activity in *FSHB* −211G>T T-allele carriers. Obviously the 2039A>G effects on testicular volume and FSH could be clearly detected only when the 2–3 times stronger influence of *FSHB* −211G>T was taken into account. Homozygous carriers of the *FSHR* 2039 G-allele had significantly higher FSH levels and lower testicular volumes in the presence of *FSHB* −211G>T GT and TT carriers. Interestingly, here again increased LH levels were observed comparable with the findings on *FSHB* −211G>T.

The question of whether carriers of *FSHB* −211G>T, and putatively *FSHR* 2039A>G or the most deleterious combinations, might benefit from FSH treatment is clinically most important but also most challenging. FSH treatment of infertile men with oligo- or even azoospermia has been widely studied, but the results are ambiguous. Not surprisingly, a recent Cochrane review (28) concluded that "the number of trials and participants is insufficient to draw final conclusions." Then again, successful treatment with FSH seems possible in some men "using some predictive parameters" (29). Now that the influence of the two SNPs in *FSHB* and *FSHR* on FSH serum levels and likely spermatogenesis are being elucidated, at least *FSHB* −211G>T TT homozygotes and possibly also heterozygotes are candidates who may benefit from FSH treatment because both have decreased FSH levels. Concerning *FSHR* 2039A>G, it is less clear whom FSH treatment may help, but the GG homozygotes appear to be the logical candidates fitting to a recent study by Selice *et al.* (30). According to our model, about 45% of men could therefore be putative responders to FSH treatment, which is close to the observed response rate in unselected men. Very recently the first treatment study taking *FSHB* −211G>T into account has been published by Ferlin *et al.* (15) with significant improvement in sperm count and quality being reported for TT homozygotes in comparison with G-allele carriers. Although the study lacked a control group and the sample size was small (only nine TT carriers), the observations fit our hypothesis well. Whether *FSHB* −211G>T, possibly in conjunction with *FSHR* 2039A>G, will become the markers for a pharmacogenetic approach to FSH treatment in male infertility, though, remains to be elucidated by larger, randomized controlled trials. To answer the clinically relevant question of an increase in pregnancy rates, such a trial would need to include at least 160 couples (assuming 50% treated/placebo, an increase in pregnancy rate from 20 to 40%, and power of 80%). Given the carrier frequency of *FSHB* −211G>T (TT homozygotes ~3%), a large number will need to be screened. Consequently, multicenter studies are necessary to achieve these goals.

Our study revealed both *FSHB* −211G>T and *FSHR* 2039A>G as promising candidates to make their way into the routine clinical workup of the male with oligozoospermia because the major prerequisites for screening procedures are met: the cause-effect relationship is becoming increasingly solid, their analysis is feasible, and, most importantly, an appropriate treatment option with FSH is available. However, first, controlled treatment studies and analyses involving other ethnic groups are warranted. By adding almost 2000 men with couple infertility to the previously published data, which signifies the clinical importance of *FSHB* −211G>T and *FSHR* 2039A>G in male infertility, we provide important justification for these studies to be performed.

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Address all correspondence and requests for reprints to: Dr. med. Frank Tüttelmann, Institute of Human Genetics, University of Münster, Vesaliusweg 12-14, 48149 Münster, Germany. E-mail: frank.tuettelmann@ukmuenster.de.

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