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Association between follicle-stimulating hormone receptor polymorphisms and reproductive parameters in young men from the general population.

Running head: FSH receptor polymorphisms in men.

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Conflicts of interest

There are no conflicts of interest.

Abstract

Objective Follicle-stimulating hormone (FSH) regulates gametogenesis through binding to its receptor (FSHR). In women, the Thr307Ala and Asn680Ser polymorphisms in the *FSHR* gene affect reproductive function, but it is not clear whether they have any impact on spermatogenesis and have mainly been investigated in infertile men of varying ages. The aim of the present study was therefore to examine whether these genetic variants of the *FSHR* influence reproductive parameters in men from the general population.

Methods Men aged 17-20 years ($n=313$) were genotyped. All men provided a semen sample and a blood sample for hormonal measurements and DNA extraction. They underwent a medical examination and analyses of possible associations between Thr307Ala and Asn680Ser polymorphisms and hormonal and sperm parameters were subsequently carried out.

Results Men homozygous for Thr307/Asn680 had a lower mean serum FSH concentration (3.07 vs. 3.65 IU/l, $P=0.009$), and higher mean serum estradiol (94.0 vs. 86.1 pmol/l, $P=0.001$), sex hormone-binding globulin (33.6 vs. 31.3 nmol/l, $P<0.0001$), and total testosterone (19.1 vs. 17.9 nmol/l, $P<0.0001$) concentrations compared with men with other genotypes. In addition, sperm concentrations (71.9×10^6 vs. 70.8×10^6 /ml, $P=0.040$) and the total sperm counts were higher (212×10^6 vs. 206×10^6 , $P<0.0001$) and their testes volumes were larger (left: 11.5 vs. 11.0 ml, $P<0.0001$; right: 12.4 vs. 11.6 ml, $P=0.002$).

Conclusion As in women, the results from the present study indicate that variants of the *FSHR* influence reproductive parameters in men.

Keywords: follicle-stimulating hormone receptor, polymorphism, sperm.

Introduction

Follicle stimulating hormone (FSH) is essential for normal reproductive function in humans. In men, FSH regulates testicular development and initiates and maintains spermatogenesis [1]. The hormone acts through its receptor (FSHR) in the cell membrane of the Sertoli cells, where it functions as a spermatogonial survival factor [2]. In women, FSH controls folliculogenesis and drives oocyte maturation. The FSHR is located in the plasma membrane of granulosa cells and regulates proliferation of these cells when stimulated by FSH [3]. An inactivating point-mutation in the *FSHR* gene (Ala189Val) has been linked to recessive hereditary ovarian dysgenesis and infertility in homozygous women [4]. Men homozygous for the same inactivating point-mutation had various fertility statuses, and yet, they were not completely infertile and they did not present with azoospermia [5]. However, naturally occurring inactivating mutations in the *FSHR* gene are rare, in contrast to single nucleotide polymorphisms (SNPs), which are common.

Two frequently studied non-synonymous SNPs in the *FSHR* gene are located in exon 10, in amino acid positions 307 and 680, respectively [6]. Both SNPs cause a replacement of adenine (A) by guanine (G), which in codon 307, results in an amino acid substitution from threonine (Thr) to alanine (Ala) and, in codon 680, causes a substitution from asparagine (Asn) to serine (Ser). The two SNPs are in linkage disequilibrium and hence yield to two predominant haplotypes, consisting of Thr307 in combination with Asn680 and Ala307 in combination with Ser680. The combination Thr307/Asn680 is present in ~60% of white populations, whereas Ala307/Ser680 is present in ~40% [6,7]. The genotype distribution of the polymorphism in position 680 differs according to ethnicity so that the homozygous Ser680 genotype is more uncommon in Asia than among Europeans, whereas the homozygous Asn680 genotype is clearly

over-represented in Asian populations [8]. Two uncommon allelic combinations, Thr307/Ser680 and Ala307/Asn680, are present in less than 5% of populations of European and Asian origin [9,10].

The functionality of the above mentioned SNPs has been studied extensively in women. In two studies on 161 and 217 women, respectively, those homozygous for Ser680 showed a higher basal serum FSH concentration and a lower sensitivity to FSH when hyperstimulated before in vitro fertilization [11,12]. Women homozygous for Ser680 also had longer menstrual cycles compared with those homozygous for Asn in the same position [13].

In men, less information is available. The allelic variants of the *FSHR* have been reported to be similarly distributed in fertile and infertile men [14-16], and no differences in sperm and testicular parameters were noted between fertile controls and infertile men, subdivided according to *FSHR* genotype [6,9,17,18]. In contrast, some studies have shown differences in the *FSHR* genotype distribution among fertile controls and infertile men [18-20]. In most studies, no differences have been observed in serum FSH concentration, serum testosterone concentration, or testicular size, the only exceptions being a study from Korea and one from Egypt. In both the Korean study, on 70 infertile men and 50 controls, and in the Egyptian study, including 52 infertile men and 30 controls of White origin, Ser/Ser in position 680 resulted in higher FSH in the circulation in the infertile men [14,16]. In a recent study from Italy, correlating the *FSHR* genotype to the response to FSH stimulation in 70 subfertile men, only men with at least one Ser in position 680 showed a statistically significant improvement in sperm parameters, indicating that men with the Ser variant may be more sensitive to FSH than counterparts with Asn in the

same position, which is in contrast to what has been shown for women [21]. However, if the FSHR variant with Ser in position 680 is less sensitive to FSH, these men would be expected to have a less effective spermatogenesis and would therefore benefit from FSH treatment. In this study, however, the age and ethnic origin of the study population were not reported and the abstinence time was not recorded. It is therefore difficult to draw firm conclusions from this study, as the ethnicity as well as the variations in age could be considered as potential confounding factors when associating FSHR variants with reproductive parameters, as basal FSH is known to increase with age [22,23].

As previous studies have mainly been based on men of varying ages attending infertility clinics, our objective was to screen young men from the general population, within a short age span, to highlight possible differences in reproductive parameters between men with different variants of the FSHR. By defining those that are genetically predisposed to benefit from FSH treatment, impaired spermatogenesis could possibly be managed in this category of subfertile men.

Materials and methods

Participants

Details on the study population have been reported elsewhere [24]. Briefly, the study population included 241 Swedish military conscripts and 73 Swedish subjects recruited through advertisements in schools or as friends of participants. They were 17-20 years of age when recruited into the study during the years 2008-2010. All study participants were of White origin.

The study participants provided a semen sample, and semen analysis was performed according to the World Health Organization guidelines from 1999 [25]. They also underwent a medical examination including determination of testicular size using ultrasound. A venous blood sample was drawn for subsequent hormonal analysis and genotyping. All men were asked to fill in a questionnaire before the medical examination, and information on abstinence time and height and length were recorded. All participants signed an informed consent form, and the study was approved by the ethical review board of Lund University.

Hormonal analysis

For endocrine serum analysis, blood samples were drawn between 8 and 10 a.m. Estradiol (E2) concentration in the serum was analyzed using an immunofluorometric method (Autodelphia; Wallac Oy, Turku, Finland) at the routine clinical chemistry laboratory at Skåne University Hospital (Malmö), whereas FSH, luteinizing hormone (LH), sex hormone-binding globulin (SHBG), and total testosterone concentrations were measured using an electrochemiluminescence immunoassay (Cobas-Roche, Mannheim, Germany) at the routine clinical chemistry laboratory

at Skåne University Hospital (Lund). The sensitivities of the assays were 8 pmol/l (E2), 0.10 IU/l (FSH), 0.10 IU/l (LH), 0.35 nmol/l (SHBG), and 0.087 nmol/l (total testosterone). The intra-assay and interassay coefficients of variations (CV) were 20% (intra-assay CV) for E2, 3.9% (intra-assay CV) and 3.9% (interassay CV) for FSH, 2.2% (intra-assay CV) and 2.3% (interassay CV) for LH, 0.9% (intra-assay CV) and 0.8% (interassay CV) for SHBG, and 0.6% (intra-assay CV) for total testosterone.

Genotyping

Genomic DNA was isolated from peripheral leukocytes using standard procedures. The *FSHR* SNPs in positions 307 and 680 in the amino acid sequence (rs6165 and rs6166 in dbSNP, respectively) were analyzed using the allele-specific PCR technique. The *FSHR* gene was amplified using two reactions for each participant, each containing one wild-type or one mutant-specific primer at concentrations of 0.12 $\mu\text{mol/l}$ as well as two flanking primers (forward and reverse, respectively) at concentrations of 0.3 $\mu\text{mol/l}$ (Invitrogen, Stockholm, Sweden; Table 1). Amplifications were performed in a total volume of 50 μl , containing, in addition to the primers, 10 mmol/l Tris-HCl pH 9.1, 45 mmol/l KCl, 0.1% w/v Tween 20, 1.5 mmol/l MgCl_2 , 200 $\mu\text{mol/l}$ of each dNTP (dATP, dCTP, dGTP and dTTP, Fermentas, Sankt Leon-Rot, Germany), 1 U Dynazyme II DNA polymerase (Finnzymes Oy, Espoo, Finland), and 200 ng template DNA. Amplifications were carried out for 26-33 cycles, each including denaturation for 1 min at 96°C, primer annealing at 57°C (rs6165) or 56°C (rs6166) for 30 s, and extension for 3 min at 72°C. An initial hotstart at 96°C and a final extension for 5 min at 72°C were also used. The control fragment and the allele-specific fragments were 549, 227 and 228 bp, respectively, for the rs6165 polymorphism, and 662 and 221 bp, respectively, for the rs6166 polymorphism. Thirty samples

representative for each genotype of both SNPs were purified and sequenced directly on an eight-capillary Applied Biosystems (Applied Biosystems, Stockholm, Sweden) sequencing gear to confirm the results from the PCR.

Statistical analysis

Calculations for associations with clinical parameters were carried out using all possible genetic models: men homozygous for the A-allele versus other genotype groups (i.e.

Thr307Thr/Asn680Asn vs. Thr307Ala/Asn680Ser and Ala307Ala/Ser680Ser), men homozygous for the G-allele vs. other genotype groups (i.e. Ala307Ala/Ser680Ser vs. Thr307Thr/Asn680Asn and Thr307Ala/Asn680Ser), and each genotype group separately (i.e. Thr307Thr/Asn680Asn vs. Thr307Ala/Asn680Ser vs. Ala307Ala/Ser680Ser). An additive model was also used, where a trend of the increased number of the G-allele was correlated with reproductive parameters.

All hormonal, sperm, and testicular residuals were tested for normal distribution in the Kolmogorov-Smirnov test, and were subsequently log transformed because of lack of normality. Comparisons of hormonal concentrations and sperm and testicular parameters, provided as mean \pm SD, among genotype groups were carried out using a univariate analysis of variance. A linear regression model was used for calculation of the additive genetic effect. BMI was considered a confounding factor (as a continuous variable) when analyzing differences in serum hormone concentrations and in testicular volumes, whereas BMI (as a continuous variable) and abstinence time (five abstinence groups: <48, 49-72, 73-96, 97-120, >120 h) were considered as confounding factors when analyzing differences in sperm parameters.

Three men were excluded from all statistical analyses because of recombinant alleles, and hence 310 men were analyzed for differences in hormone concentrations in relation to *FSHR* genotype, as well as differences in testicular volumes. In addition, sperm parameters were not available for all men; thus 293 men were included when analyzing differences in sperm concentrations and progressive sperm motility, whereas 294 men were included when analyzing differences in the total sperm count. Data were analyzed using PASW software version 18 (SPSS Inc., Chicago, Illinois, USA). A *P*-value of less than 0.05 was considered significant.

Results

Allele frequency and genotype distribution

In Table 2, the allele frequencies and the genotype distribution of Thr307Ala and Asn680Ser in the 313 men investigated are presented. The distribution of the two SNPs was similar and confirmed a link between Thr307 and Asn680 as well as between Ala307 and Ser680; men homozygous for Thr307 were also homozygous for Asn680, whereas men homozygous for Ala307 concomitantly were homozygous for Ser680. Heterozygous men were heterozygous in both positions. R^2 in the study population was 0.99.

Of the 313 study participants, three men (1%) had recombinant alleles, of whom two had Thr307Thr/Asn680Ser and one had Thr307Ala/Ser680Ser. Two of these men (one of each combination) had fathers descended from Chile.

Hormone concentrations

A significant association was found between the Thr307Thr/Asn680Asn genotype and a higher mean serum E2 concentration compared with other genotype groups ($P=0.001$; Table 3). Men with the Thr307Thr/Asn680Asn genotype also had a significantly lower mean serum FSH concentration compared with other genotype groups ($P=0.009$), as well as a significantly higher mean serum SHBG concentration ($P<0.0001$) and a significantly higher mean serum testosterone concentration ($P<0.0001$). However, no significant relationship was found between genotype groups and mean serum LH concentration ($P=0.141$). An additive effect of the G-allele was observed in E2 ($P=0.001$) and FSH ($P=0.036$).

Sperm and testicular parameters

Men with the Thr307Thr/Asn680Asn genotype had a marginally higher mean sperm concentration compared with the other genotype groups ($P=0.040$; Table 3), but only when the other two genotype groups were treated as one group. Men with the Thr307Thr/Asn680Asn genotype also had significantly higher total sperm counts compared with other genotype groups ($P<0.0001$), as well as significantly larger testes than heterozygous counterparts and counterparts with the Ala307Ala/Ser680Ser genotype (left: $P<0.0001$; right: $P=0.002$). No difference was found in progressive sperm motility between genotype groups ($P=0.967$). An additive effect of the G-allele was found in testis size on the left side (left: $P=0.021$; right: $P=0.055$).

Of the three men with recombinant alleles, two (one with Thr307Thr/Asn680Ser and one with Thr307Ala/Ser680Ser) had spermatocele on the left testicle, compared with nine men (3%) in the entire study population (left or right testicle). All other reproductive parameters were within the normal range.

Discussion

The main finding of the present study was that in young men from the general population, those homozygous for the A-allele (Thr307Thr/Asn680Asn) had lower serum FSH concentration, higher serum E2, SHBG, and total testosterone concentrations compared with carriers of other FSHR variants. In addition, they had higher sperm concentrations, total sperm count, and larger testicles compared with counterparts with other genotypes. These findings are in agreement with those found previously in women [11,12] and in some previous reports on men [14,16], although the men in those reports were infertile or a mixture of infertile and fertile men of different ages.

The lower serum FSH concentrations in the Thr307Thr/Asn680Asn group could indicate a more active FSHR, requiring a lower amount of FSH to generate a downstream intracellular signal in the FSH signaling pathway that is similar to the signal generated by a less active receptor in combination with a higher serum FSH concentration. A higher serum E2 concentration could also indicate a more active FSHR in these individuals, as aromatase, which is the enzyme that converts testosterone into E2, is expressed in Sertoli cells as a downstream response to FSH stimulation [26]. The higher SHBG concentration observed in this group of men might be attributed either to a more active FSHR, as FSH also regulates the production of testis-produced SHBG, sometimes called androgen-binding protein, in the Sertoli cells [27] or to the increased levels of E2.

A higher SHBG concentration would consequently result in a lower amount of bioavailable testosterone in the circulation, which, through the negative feedback loop to the pituitary, would lead to higher LH and subsequently induced testosterone secretion. Indeed, carriers of

Thr307Thr/Asn680Asn presented with higher total testosterone as well as LH concentrations compared with men with other genotypes, although the LH concentration was not statistically significantly different.

The higher sperm concentrations, total sperm count, and the larger testicles observed in the Thr307Thr/Asn680Asn group could similarly be the results of a more active FSHR, which, in combination with a higher testosterone concentration, would increase spermatogenesis. Because of the young age of the men included in the current study, we cannot draw any conclusions on fertility in relation to the *FSHR* genotype. However, the difference in the mean sperm counts found between the two groups was marginal and may not necessarily imply any discrepancies in fertility. Nevertheless, it was remarkable that the FSH levels were almost 20% higher in the Thr307Ala/Asn680Ser and Ala307Ala/Ser680Ser group, which might indicate a need for genotype-adjusted reference values for FSH when evaluating FSH as a marker of reproductive function in men.

The mechanisms underlying the higher activity of the FSHR found in men homozygous for the Thr307Thr/Asn680Asn genotype are not known, but could possibly be attributed to an altered glycosylation status of the FSHR, as Asn may introduce a potential glycosylation site in amino acid position 680 [28]. Glycosylation is important for post-translational protein processing and expression of the FSHR at the cell surface, which has been shown in a previous study, where a glycosylated Asn either in position 174 or in position 276 was required for efficient folding of the FSHR in order to promote high binding affinity of FSH [29]. An altered phosphorylation status of the FSHR could also be expected, as Thr in position 307 and Ser in position 680 may

introduce phosphorylation sites that might affect the FSH-binding capacity (position 307) or the downstream intracellular signaling of FSH stimulation (position 680). Previous studies have confirmed the region of the FSHR enclosing position 307 to be important for binding of FSH to the FSHR [30,31], and hence, an amino acid shift in this region may affect signal transduction and receptor activity. In other species where the amino acid sequence of the FSHR have been cloned, for example the non-human primate *Macaca fascicularis* [32], cow [33], horse [34], and sheep [35], position 680 is occupied by Asn, indicating that the Asn allele may be the ancestral one.

As multiple testing was performed, the issue of mass significance was considered, but as eight of 10 possible associations were found to be statistically significant, no correction was performed and thus the results need to be confirmed in an independent study population.

Conclusion

Young men homozygous for Thr307 and Asn680 have lower serum FSH concentrations, higher serum E2, SHBG and total testosterone concentrations, slightly higher sperm concentrations, higher total sperm count and larger testicles than heterozygous subjects and subjects homozygous for Ala307 and Ser680. It remains to be elucidated whether this genetic variation has an impact on fertility status and if genotype-adjusted FSH reference values for evaluation of men from infertile couples need to be established.

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Table 1 **Primer sequences**

Primer direction	Thr307Ala	Asn680Ser
Forward (flanking)	5'-TAGCCTCAAGGGCAGGTATG-3'	5'-TTCACCCCATCAACTCCTGT-3'
Reverse (flanking)	5'-GATGCAATGAGCAGCAGGTA-3'	5'-TCCTGGCTCTGCCTCTTACA-3'
Reverse (allele-specific A)	5'-GAGGATCTCTGACCCCTAGT-3'	5'-GACAAGTATGTAAGTGGAAACCAT-3'
Reverse (allele-specific G)	5'-AGGATCTCTGACCCCTAGC-3'	5'-GACAAGTATGTAAGTGGAAACCAC-3'

Table 2 **Frequencies and distribution of Thr307Ala and Asn680Ser**

	Allele frequencies (%)		Genotype distribution [% (n)]		
	A	G	AA	AG	GG
Thr307Ala	55	45	28.7 (90)	53.4 (167)	17.9 (56)
Asn680Ser	55	45	28.1 (88)	53.7 (168)	18.2 (57)

1 **Table 3** Comparisons of serum hormone concentrations, sperm parameters and testis volumes among genotype groups

	TT/NN		TA/NS		AA/SS		P-value			Trend
	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	<i>n</i>	Mean (SD)	TT/NN vs. TA/NS vs. AA/SS	TT/NN vs. TA/NS, AA/SS	TT/NN, TA/NS vs. AA/SS	
E2 (pmol/l)	88	94.0 (24.3)	166	87.4 (22.8)	56	82.1 (22.7)	0.001	0.001	0.004	0.001
FSH (IU/l)	88	3.07 (1.36)	166	3.69 (2.15)	56	3.54 (1.58)	0.025	0.009	0.152	0.036
LH (IU/l)	88	4.44 (1.27)	166	4.93 (1.77)	56	4.59 (1.64)	0.099	0.141	0.275	0.654
SHBG (nmol/l)	88	33.6 (10.6)	166	31.1 (11.6)	56	32.0 (9.96)	<0.0001	<0.0001	<0.0001	0.504
T (nmol/l)	88	19.1 (5.37)	166	18.1 (5.17)	56	17.5 (4.73)	<0.0001	<0.0001	<0.0001	0.107
Progressive sperm motility (%)	84	52.2 (17.9)	158	52.4 (16.7)	51	52.6 (17.5)	0.991	0.967	0.990	0.808
Sperm concentration ($\times 10^6$/ml)	83	71.9 (61.8)	159	69.0 (53.1)	51	76.5 (79.7)	0.081	0.040	0.040	0.956
Total sperm count ($\times 10^6$)	83	212 (231)	159	208 (209)	52	201 (221)	<0.0001	<0.0001	<0.0001	0.923
Testis volume, left (ml)	88	11.5 (3.95)	166	11.2 (3.19)	56	10.2 (3.22)	<0.0001	<0.0001	<0.0001	0.021
Testis volume, right (ml)	88	12.4 (4.28)	166	11.8 (3.58)	56	11.1 (3.01)	0.003	0.002	0.002	0.055

- 2 A, Ala; E2, estradiol; FSH, follicle-stimulating hormone; LH, luteinizing hormone; N, Asn; S, Ser; SHBG, sex hormone-binding
- 3 globulin; T, Thr.