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Article type : Original Article

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FSHB and *FSHR* gene variants exert mild modulatory effect on reproductive hormone levels and testis size but not on semen quality: A study of 2,020 men from the general Danish population.

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Short title: *FSHB* and *FSHR* genetic variants and testicular function.

Key terms: *FSHB*, *FSHR*, FSH, male infertility, testicular function.

Word count: 3542. Number of figures and tables: 1 figure & 5 tables (5 supplementary tables)

Abstract

Background: Spermatogenesis depends on stimulation by follicle-stimulating hormone (FSH) which binds to FSH receptors (FSHR) on testicular Sertoli cells. Three FSH-related single nucleotide polymorphisms (SNPs); *FSHB* -211G>T (rs10835638), *FSHR* -29G>A (rs1394205) and *FSHR* 2039A>G (rs6166) affect FSH action, and have been suggested to affect testicular function, but the evidence is uncertain.

Objective: To describe the associations between the three SNPs and testicular function in a large and well-characterised cohort of men from the general population.

Materials and methods: A cross-sectional study of 2,020 Danish men unselected regarding testicular function. Outcome variables were semen parameters, reproductive hormones and testis size. Genotyping was done by competitive allele-specific quantitative PCR. Differences in genotype frequencies were tested by Chi-square test and associations between genotypes and outcomes were assessed by multivariate linear regressions.

Results: The SNPs affected serum FSH; carriers of the variant affecting FSH secretion (*FSHB* -211G>T) had lower FSH levels while carriers of variants affecting receptor expression (*FSHR* -29G>A) and receptor sensitivity (*FSHR* 2039A>G) had higher FSH levels. Carriers of *FSHB* -211G>T had lower calculated free-Testosterone/LH ratio. Although both *FSHB* -211G>T and *FSHR* 2039A>G were associated with smaller testis size, no clear association was detected in relation to any semen parameters, except a lower total number of morphologically normal spermatozoa in the heterozygous carriers of the *FSHB* -211G>T

Discussion and Conclusion: The studied polymorphisms have only minor modulating influence on testis size and function in healthy men. We detected subtle effects of the three SNPs on FSH levels, but also effects of *FSHB* -211G>T on calculated free-Testosterone/LH ratio, compatible with altered Leydig cell function. Thus, the role of these FSH-related polymorphisms is complex and modest in men with normal testicular function, but the possible importance of FSH polymorphisms in men with impaired testicular function should be evaluated in future studies in more detail.

Introduction

Spermatogenesis can be affected by multiple factors including genetic variations modulating the function of the hypothalamus-pituitary-gonadal (HPG) hormone axis[1,2] as well as environmental and lifestyle factors[3,4].

Single nucleotide polymorphisms (SNPs) in several genes have been associated with male infertility and decreased semen quality[1,2]. SNPs that affect the follicle stimulating hormone (FSH) signalling pathway essential for normal spermatogenesis, have gained attention[5–9]. Particularly, the *FSHB* -211G>T SNP, located in the promoter region of the FSH beta-subunit, influences the transcription of the *FSHB* gene and consequently circulating FSH levels[10]. Minor T-allele carriers of *FSHB* -211G>T have lower serum levels of FSH and inhibin B and smaller testicular volume[10–15]. Furthermore, two FSH receptor (FSHR) SNPs, *FSHR* -29G>A and *FSHR* 2039A>G, have been linked to testicular function by affecting the transcription of FSHR and the sensitivity of FSHR, respectively[14–19]. In some studies, the minor alleles of these three SNPs were found to be more prevalent among infertile men than among men from the general populations and proven fathers[11,13,20], the *FSHR* SNPs in combination with the *FSHB* -211G>T *SNP* have been shown to result in a more severe phenotype[14]. However, other studies found no effect of especially the *FSHR* variants on male reproduction suggesting that the HPG axis may compensate for the effects of these genetic variants in some men[9,21]. Here, we address this question by investigating the three most informative SNPs: *FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G on gonadal function, in a very large group of well-characterised young Danish men from the general population.

Material and Methods

Study population

A total of 2,476 men who participated in a study of testicular function in the period 2007-14 were considered for inclusion in the current study. The men, median age 19 years, were invited on the day of a compulsory medical examination to determine their fitness for military service as described in details previously[22,23]. On the day of participation, they handed in a questionnaire, underwent a physical examination, had a blood sample drawn, and delivered a semen sample. All participants were asked to abstain from ejaculation for at least 48 hours. The actual abstinence

period was recorded. The participants received 500 DKK (~ 67 Euro) in compensation for their participation. Of the 2,476 participating men, 456 were excluded from the current study due to missing DNA samples (n=234), use of anabolic steroids self-reported or indicated by their hormone profile from blood samples (n=21), having parents of non-Danish origin (n=176), chronic or newly diagnosed serious disease (n=13), serious testicular injury or major surgery (n=10) or azoospermia (n=2), leaving 2,020 men eligible for inclusion in the current study.

The 2,020 men were stratified into two subgroups; subgroup A without any known factor that potentially could impair testicular function (n=1,419) and subgroup B (n=601) with the presence of one or more factors that could potentially affect the testicular function i.e. varicocele, treated cryptorchidism, previous testicular infections, previous minor scrotal injury or surgery (Table 1). Reproductive parameters, clinical description and medical history for the total population and in subgroup A and B respectively are summarised in Table 1.

Physical examination

All men underwent a physical examination by trained physicians. Testis size was measured by ultrasound. Body weight and height were measured and body mass index (BMI) calculated.

Reproductive hormone assessment

Blood samples were drawn from the cubital vein between 8.00 and 12.15 a.m. (median time 09.55 a.m.). Serum samples were separated and frozen at -20 °C until analysed in batches. Serum levels of FSH, luteinizing hormone (LH), and sex hormone-binding globulin (SHBG) were determined using a time-resolved immunofluorometric assay (DELFIA; Wallac Oy, Turku, Finland) except the samples from 2014 where SHBG was determined by ELISA (Access2, Beckman Coulter Ltd., High Wycombe, UK). Total testosterone and estradiol levels were initially determined using time-resolved fluoroimmunoassays (DELFIA; Wallac Oy) but, from 2010, estradiol was measured with radioimmune analysis (Pantex, Santa Monica, CA) and from 2014, total testosterone was measured by ELISA (Access2, Beckman Coulter Ltd., High Wycombe, UK). Free testosterone was calculated assuming a fixed albumin value of 43.0 g/L using the calculation suggested by Vermeulen *et al.*[24]. Inhibin B was determined by a specific 2-sided enzyme-immunometric assay (Oxford Bio-Innovation Ltd and Inhibin B Gen II; Beckman Coulter Ltd., High Wycombe, United Kingdom and Serotec, United Kingdom). The reproductive hormones were measured in thawed

samples in June 2010 and December 2013 through January 2014. One-hundred samples from 2010 were re-analysed in 2013/2014 to ensure comparability of the hormone levels.

Semen analysis

All men produced semen samples by masturbation on site. Semen analysis was conducted as previously described using methods in accordance with the most recent WHO recommendation[22,23,25]. Semen volume was assessed by weighing. Sperm concentration was assessed using a Bürker-Türk haemocytometer. For sperm motility assessment, the spermatozoa were classified as either progressively motile, non-progressively motile or immotile. Papanicolaou stained smears were assessed according to 'strict criteria' to determine the number of morphologically normal spermatozoa[26]. Total sperm counts were calculated as semen volume x sperm concentration; total progressive sperm counts as the percentage of progressively motile sperm x total sperm count; and total morphologically normal spermatozoa as the percentage of morphologically normal sperm x total sperm count.

Genotyping

Isolation of DNA from blood samples and genotyping were done as previously described[27,28]. In brief, genotyping was performed with KASP™ genotyping assays (LGC Genomics, Hoddesdon, UK). KASP™ genotyping assays were designed by LGC Genomics against the following sequences:

FSHB -211G>T (rs10835638): TATCAAATTTAATTT[G/T]TACAAAATCATCAT;
FSHR -29G>A (rs1394205): TCTCTGCAAATGCAG[A/G]AAGAAATCAGGTGG;
FSHR 2039A>G (rs6166): ATGTAAGTGGAACCA[C/T]TGGTGACTCTGGGA.

The nucleotide sequences were obtained from GeneBank: FSHB (GenBank Gene ID: 2488), FSHR (GenBank Gene ID: 2492).

A total of 1,073 men had their genotypes analysed by LGC Genomics in Hoddesdon, UK. The remaining samples (N=947) were analysed using the same methodology and primers but at the Department of Growth and Reproduction, Rigshospitalet. Quantitative endpoint PCR was performed according to the manufacturers recommendation with a standard touch-down PCR program incubation at 94°C for 15 min., 10 cycles with 94°C for 20 s, 61-55°C for 60 s (drop

0.6°C per cycle), and 26 cycles with 94°C for 20 s, 55°C for 60 s) and resulted in clear genotype clusters for all assays.

A continuous quality assurance programme including more than 500 samples analysed in both laboratories has shown a 100% concordance between the results obtained in the two laboratories. In addition, all samples within homozygous minor allele clusters were re-analysed, and in all cases, the initial results were verified. Distributions of the three SNPs were consistent with Hardy-Weinberg equilibrium for both the total group (*FSHB* -211G>T, p=0.296, *FSHR* -29G>A, p=0.412, *FSHR* 2039A>G, p= 0.470), subgroup A (*FSHB* -211G>T, p=0.995, *FSHR* -29G>A, p=0.916, *FSHR* 2039A>G, p= 0.887), and subgroup B (*FSHB* -211G>T, p=0.437, *FSHR* -29G>A, p=0.919, *FSHR* 2039A>G, p= 0.228).

Statistics

For descriptive statistics, medians and 5–95th percentiles or percentages (%) were calculated. Differences in distributions between the two subgroups were compared by Chi-square test for categorical variables and Mann-Whitney U-test or multivariate linear regression analyses for continuous variables. Genotype frequencies between the two groups as well as results from previous studies from the Baltic countries were compared using Chi-square test.

Multivariate linear regression analyses were performed to test the association between the genotypes and reproductive parameters (semen parameters, reproductive hormones, testis size) and to test for interactions. This was performed to test the impact of the SNPs individually and the combination of all three SNPs. To meet model assumptions of normally distributed residuals and variance stability, some variables were transformed before entering the model; hormone variables were transformed by natural logarithm and semen parameters by cubic root transformation. Covariates in the statistical model included factors associated with levels of reproductive hormones and semen quality (age, fever recent three months, BMI, smoking status, use of cannabis, blood sampling hour (only for hormones), ejaculation abstinence (only for semen parameters) and duration from ejaculation to analysis of sperm motility (only for sperm motility). All models were performed with and without adjustment for covariates for all outcome variables.

Finally, we stratified men according to the 5th, 25th, 50th and 75th WHO reference centiles of the various semen parameters to analyse whether minor-allele carriers were accumulated in the lower centiles.

An additive model is shown in Supplementary Table 1-3, but to simplify the presentation of the results, we used a dominant model (aggregating heterozygotic and homozygotic minor allele carriers) in Table 4 and Supplementary Table 4.

For the figures, we combined all three genotypes into 27 combinations. Due to the low number of men in some groups, statistical analyses were not performed on these.

$P<0.05$ was considered statistically significant. SPSS version 25 for Windows was used for all statistical analyses.

We chose not to correct for multiple testing because correction for multiple testing places an unreasonable emphasis on type I statistical error while inflating the type II error that we find is more important here, considering the small sample sizes within many of the strata.

Ethical approval

The study was approved by the local science ethical committee (H-KF-289428). All men participating in the study have signed an informed consent.

Results

Basic description of the total study population (total) and two subgroups (A: without factors potentially affecting testicular function and B: with one or more such factors) are shown in Table 1.

Genotype and allele frequencies

Table 2 shows the genotype and allele frequencies of the three FSH-related SNPs in the total population and the two subgroups. There was a small, but statistically significant difference between subgroups in the distribution of the two alleles for the *FSHB* -211G>T with a lower

frequency of the minor allele (T) in subgroup A (16%) compared to B (18.6%) (p=0.045). No significant differences were detected for the two *FSHR* SNPs.

When comparing the Danish genotype- and allele frequency with previous reported results from men from the Baltic countries, results showed that Danish men have a slightly higher minor allele frequency for two of the three SNP's: *FSHB* -211G>T: 16% vs 12.9%[12], p=0.002, *FSHR* -29G>A: 26.8% vs 25.4%[15] p=0.257, and *FSHR* 2039A>G: 45.3% vs. 40%[19], p<0.001.

Semen parameters and reproductive hormones

Table 3 shows the genotype and allele frequencies of the three FSH-related SNPs stratified according to median levels of FSH, Inhibin B and testis size, as well as to the WHO criteria for normal levels of total sperm count, in subgroup A. Unadjusted associations between semen parameters, testis size and reproductive hormones according to the three SNPs in combination (dominant model) are shown in Table 4. The estimated effects of the three individual SNPs on testicular function adjusted for covariates are shown in Table 5. Supplementary Tables 1-3 show the parameters stratified according to the three individual SNPs.

As seen in Table 3-5, *FSHB* -211G>T showed the strongest associations to FSH levels. Significantly lower FSH levels were detected in both heterozygous and homozygous minor-allele carriers (T) of *FSHB* -211G>T (Table 5, p<0.001) and with a higher percentage of T-allele carriers in the men with FSH levels below median FSH (Table 3, p <0.001). For the two *FSHR* SNPs, minor-allele carriers seemed to have slightly higher levels of FSH, but the difference was only statistically significant in relation to the heterozygous (AG) men of *FSHR* -29G>A (p=0.03) and homozygous (GG) carriers of *FSHR* 2039A>G (p=0.026) (Table 5). In these groups, inhibin B levels tended to be lower, but the difference was not statistically significant. However, for *FSHB* -211G>T, a higher percentage of T-allele carriers in the group of men with Inhibin B below the median was detected (p=0.007). Conversely, the inhibin B/FSH-ratio was higher for the carriers of *FSHB* -211G>T and lower for the carriers of the two *FSHR* SNPs indicating a better primary spermatogenic capacity.

A negative association with testicular size was observed for both *FSHB* -211G>T and *FSHR* 2039A>G with a significantly higher percentage of the minor allele carriers among men with testis size below the median for *FSHB* -211G>T (p=0.013) and *FSHR* 2039A>G (p=0.024), respectively. However, no clear effect on the semen parameters were detected for either of the three SNPs. A higher percentage of the minor allele carriers of *FSHR* -29G>A was detected in the group of men with total sperm count <39 million (p=0.008) though the effect estimates were nonsignificant. For all three SNPs, there were no clear or consistent effects on total sperm count, the total progressively motile sperm number or the total morphologically normal spermatozoa. The only significant finding was the lower total number of morphologically normal spermatozoa in the heterozygote carriers of *FSHB* -211G>T (p=0.043). Similar analyses in subgroup B did not indicate more pronounced effects of the SNPs (Supplementary Tables 4 and 5).

We combined the three SNPs in the 27 genotype combinations. Figures 1A, B and C show boxplots for the FSH levels, total sperm count and testis size stratified according to the genotype combinations and Figure 2 illustrates the percentages of men in each the genotype combinations stratified according to WHO reference levels for total sperm count (≥39 million). Although, it could seem like there was a tendency of a modifying effect of the two *FSHR* variants on *FSHB* GG and GT-genotypes with a decline in the number of men with normal sperm count, the decline was not seen in the last nine groups (TT-carriers). Due to the low numbers in several groups, further statistical analysis was not possible. Stratifying men according to the 5th , 25th , 50th and 75th WHO reference centiles[25] of the various semen parameters did not show that minor-allele carriers were accumulated in the lower centiles (data not shown).

Stratifying men according to WHO reference levels of sperm motility (≥32% motile) or morphology (≥4%) did not show any clear tendency either (data not shown).

No differences in either serum testosterone, LH or calculated free testosterone levels were observed according to genotypes (data not shown), but the testosterone/LH-ratio tended to be lower in all the minor allele carriers. Also, for *FSHB* -211G>T, the ratio of calculated free T/LH was significantly lower in the heterozygous group (p=0.006) and in the homozygous SNP group (p=0.019). No associations between estradiol levels and any genotype constellations were detected (data not shown).

Discussion

We investigated the impact of three FSH-related genetic polymorphisms (*FSHB* -211G>T, *FSHR* -29G>A, and *FSHR* 2039A>G) on semen quality, reproductive hormone levels and testicular volume in a sufficiently large and well-characterised group of Danish men. All three genetic variants showed association with FSH levels and testis size, but we did not detect any clear association with semen quality. We also detected possible effect of the *FSH*/*FSHR* SNPs on the testosterone/LH ratio, which mainly depends on the Leydig cells function. Our results support a link between the three SNPs and reproductive function, but the role of these SNPs may be more complex than proposed in previous studies.

The *FSHB* -211G>T has been proposed to exert the strongest effect on testicular function[15] as the minor allele (T) has been suggested to modulate the gene transcription of the *FSHB* gene [5,8]. Our data support previous studies by showing a strong association between the T-allele and lower FSH levels[10–14,29]. Some studies have shown a link between *FSHB* -211G>T and semen quality[12–14]. However, we did not detect any significant association between the T-allele and total sperm count, but GT carriers had significantly lower total number of progressively motile spermatozoa than men with the GG genotype. This effect was not seen in the TT-group perhaps due to the small number of men with this genotype.

Though most SNPs are assumed to act in an additive manner, the results from the additive model used on the individual SNPs were unclear. Therefore, we used a dominant model, aggregating heterozygotic and homozygotic minor allele carriers, when stratifying the reproductive parameters according to genotypes and when showing the effect estimates. This simplified the combined description of all three SNPs.

We did not correct for multiple comparisons testing. Although such correction would protect against type I error, it would also deflate the type 2 error. Furthermore, the statistical tests are inter-related and multiple comparison correction is not straight forward. Anyhow, most of the statistical test results can be regarded as non-significant irrespective of correction for multiple testing.

Testis size was measured by ultrasound instead of manual palpation, because it is more accurate and enabled the detection of small differences in an otherwise generally healthy population.

The *FSHR* 2039A>G SNP in exon 10 of the *FSHR* has been well-studied in male populations[14,16–19,30–41]. The resulting amino acid change is suggested to alter the receptor sensitivity[42] and has been associated with higher FSH and lower inhibin B levels and smaller testis volume in men[14,16,18,19], in accordance with the higher FSH level and lower testis size observed in our study. Only one previous study found a clear association between *FSHR* SNPs with sperm concentration and total sperm count[18]. The promoter SNP *FSHR* -29G>A has been suggested to reduce the receptor expression[7], but only few studies have shown an effect on reproductive parameters in men[15,17,43]. Grigorova and colleagues found that carrying both the *FSHR* 2039A>G and *FSHR* -29G>A SNPs had a cumulative effect on FSH, inhibin B level and testis volume[15]. In our study, both *FSHR* SNPs were associated with higher levels of FSH accompanied by smaller testis size, but we did not find any major impact on semen parameters. Besides the effect on spermatogenic parameters, several studies have found an association between the minor alleles and lower testosterone levels alone or in combination with higher LH levels indicating an effect on Leydig cell function[12,14,18,19]. In accordance, we found that minor allele carriers of both *FSHB* -211G>T and *FSHR* 2039A>G had lower calculated free Testosterone/LH ratio as a proxy for altered Leydig cell function.

Few studies have detected a higher frequency of the three SNPs in infertile/oligo-azoospermic populations[11,13,14,17,44]. We were curious whether the relatively poor semen quality reported in the general Danish population could be at least partly explained by a distribution of the FSH-related genotypes. We compared our data with the frequency of these genotypes in men from the Baltic countries, who have on average better semen quality than the Danes[22]. Interestingly, we found a slightly higher minor allele frequency in the Danish than in the Baltic men for all three SNP's, though only significant for the *FSHB* -211G>T and *FSHR* 2039A>G SNP.

A major unanswered question is how the effects of the FSH-SNPs should be interpreted. Sperm output is regulated by numerous genes and can be affected by a plethora of external factors. Although FSH has been known for decades to stimulate spermatogenesis, it is still being investigated to what extent this gonadotropin is needed for spermatogenesis in humans [45]. Only

few cases of inactivating *FSHB* or *FSHR* mutations in men have been described and with large variations in both phenotype and spermatogenesis [46–50]. However, FSH is important for Sertoli cell development during fetal life, mini puberty and pubertal maturation[51]. *FSHB* -211G>T and *FSHR* -29G>A have also been associated with hormone levels during mini-puberty and pubertal onset in girls [28,52,53] and it can be speculated if these SNPS actually have an impact before adulthood.

In the current study, we have detected a possible effect of the *FSH*/*FSHR* SNPs on the testosterone/LH ratio, implicating possible compensatory cross-reaction between the FSH/FSHR and LH/AR pathways. Of note, in knock-out mouse studies, a negative impact of *Fshr* ablation on Leydig cell numbers and testosterone production has been detected[54,55].

It remains to be elucidated if these SNPs play a larger role in adult men with impaired capacity for spermatogenesis who thus have a higher risk of sub-fertility. It can be speculated that such men would benefit from an additional FSH stimulation. Our study did not have the power to prove this assumption because the cohort mainly contained healthy young men. In healthy men with well-developed testicles and with a good potential for spermatogenic capacity, small variations in FSH activity could have little or no effect on sperm output. We did not observe any increased effect even in subgroup B, who had lower testis function and higher compensatory FSH levels, albeit most of these men did not have reduced semen quality. A recent study investigated the potential effect of *FSHB*-211G>T on the Sertoli cells and found no association between the genotypes and the Sertoli cell number[56]. Other studies have shown that FSH treatment can improve semen parameters in infertile men carrying minor alleles of *FSHB* -211G>T and *FSHR* 2039A>G[13,57,58]. Large intervention studies of FSH treatment of infertile men are needed to elucidate if these SNPs could be used as genetic markers for prediction of response to treatment[59].

In conclusion, our study investigated the combined effects of three FSH-related SNPs. The findings of the present study are overall in agreement with previously published studies. Our results do not support a major impact of the FSH-related SNPs on testicular function in men with largely normal testis function. A minor modulating influence of the genotype distribution on the average parameters of the testis function at the population level is possible, though at the individual level the effects of these genotypes on testis function are apparently subtle and likely influenced by other factors in healthy young men.

Acknowledgements

We owe a special thanks to all the volunteers who participated in the study. We also thank everyone at the Department of Growth and Reproduction who participated in data collection and analyses and especially the staff of molecular biology laboratory.

Fundings

This work was supported by the Faculty of Health and Medical Sciences, University of Copenhagen, The Innovation Fund Denmark (grant number 14-2013-4), Danish Council for Independent Research (DFF-1331-00044) and the Novo Nordisk Foundation (R195-A16270).

Declaration of interest

The funding institutions had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. None of the authors have any competing interests to declare.

Author contributions

Substantial contributions to conception and design: AKB, NJ, ERM, KA, LN and LP. Data acquisition: AKB, LN, LP, MBJ, MK, SAH, DLEP, SBW, UNJ, IAO, HWH and KA, Data analysis: AKB, NJ, KA and JHP. Data interpretation: All authors. Drafting the manuscript and critical discussion: AKB and NJ. Revising manuscript critically for important intellectual content: All authors. Final approval of the manuscript: All authors.

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Legends to Figure 1:

Boxplots of FSH levels (A), Total sperm count (B) and Average testis size (C) for each genotype combination. The boxes represent the 25th-75th percentiles (interquartile range) with the median, and whiskers show the highest and lowest value that are within 1.5 times the interquartile range. Outliers, defined as observations below or above these values, are not depicted in the figure. There were no men in group 26, indicated in grey.

Legends to Figure 2:

Percentage of men with a normal total sperm count (≥39 million) in each of the 27 genotype combinations (*FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G) in the subgroup (A) of men without any known factors that could potentially affect the testicular function.

Table 1: Basic description of the whole study population (total) and stratified into Subgroup A (without factors potentially affecting testicular function i.e. varicocele, treated cryptorchidism, previous testicular infections, previous minor scrotal injury or operations) and subgroup B with one or more such factors. Values are medians (5-95th percentiles) or percentage.

	N	Total (N=2,020)	Subroup A (N=1,419)	Subgroup B (N=601)	p-value
Physical appearance					
Age (years)	2,015	19 (18-22)	19 (18-22)	19 (18-22)	0.257 #
Height (cm)	1,985	182 (172-193)	182 (171-193)	182 (173-193)	0.166 #
Weight (Kg)	2,007	74 (59-94)	74 (59-94)	74 (60-91)	0.223 #
Body Mass Index (kg/m2)	1,974	22 (18-28)	22 (18-28)	22 (19-27)	0.499 #
Average testis size, ultrasound (ml) (a)	1,921	13.5 (8.3-20.7)	13.5 (8.5-20.8)	13.4 (8.1-20.3)	0.296 #
Varicocele (b)	1,969	15.3%	0.0%	50.8%	**0.000 ¤**
Lifestyle					
Alcohol > 14 units recent week	2,019	27.8 %	28.0%	34.9%	0.745 ¤
Smoking , daily	2,013	31.0%	29.4%	35.0%	0.068 ¤
Any medication the last 3 months (c)	2,008	25.4%	24.4%	27.9%	0.097 ¤
Cannabis at least once the last 3 months	1,721	41.3%	41.1%	41.5%	0.875 ¤
Previous diseases or been treated for					
STD, epididymitis, or orchitis (d)	2,002	6.6%	0.0%	22.4%	**<0.001 ¤**
Treated cryptorchidism (e)	2,019	2.4%	0.0%	8.0%	**<0.001 ¤**
Scrotal injury (f)	1,998	8.4%	0.0%	28.0%	**<0.001 ¤**
Scrotal operations (g)	2,019	2.3%	0.0%	7.7%	**<0.001 ¤**
Hormones					
FSH (IU/l)	1,999	2.5 (1.0-6.2)	2.5 (0.9-5.9)	2.6 (1.0-6.9)	**0.015 #**
Inhibin B (pg/ml)	1,996	169 (82-284)	172 (84-289)	162 (77-268)	**0.002 #**
Inhibin B/FSH	1,996	69 (15-255)	70 (17-258)	66 (13-233)	**0.002 #**
LH (IU/l)	1,999	3.4 (1.6-6.6)	3.4 (1.6-6.5)	3.3 (1.6-7.0)	0.259 #
Testosterone (nmol/l)	1,998	20.0 (11.9-32.8)	19.8 (11.9-33.0)	20.3 (11.8-32.1)	0.358 #
Testosterone/LH	1,998	6.0 (2.9-12.2)	5.9 (2.9-12.1)	6.0 (2.9-12.4)	0.696 #
Free testosterone (pmol/l) (h)	1,996	445 (269-740)	445 (271-742)	446 (262-730)	0.090 #
Sex hormone-binding globulin (nmol/l)	1,997	30 (15-55)	30 (15-55)	31 (16-56)	0.262 #
Estradiol (pmol/l)	1,998	80 (46-136)	81 (46-137)	80 (48-133)	0.324 #
Semen					
Ejaculation abstinence (hrs)	2,014	62 (37-135)	62 (37-134)	62 (37-142)	0.606 #
Semen volume (ml)	2,015	3.3 (1.3-6.3)	3.2 (1.3-6.2)	3.4 (1.3-6.3)	0.298 #
Sperm concentration (mill/ml)	2,015	46 (4-159)	48 (5.5-161)	41 (3.1-158)	**0.001 #**
Total sperm count (mill)	2,006	143 (14-534)	146 (18-552)	134 (10-510)	**0.002 #**
Progressive motile spermatozoa (%)	2,007	59 (25-79)	59 (26-80)	59 (23-78)	0.848 #
Morphologically normal spermatozoa (%)	1,926	6.5 (0.5-16.0)	6.5 (1.0-16.0)	6.5 (0.5-16.0)	0.299 #
Normozoospermic (%) (i)	2,005	63.6%	64.0%	61.6%	0.318 ¤
Oligozoospermic (%) (j)	2,015	15.8%	14.5%	18.9%	**0.015 ¤**

a: Mean of left and right testicles assessed by palpation, b:Varicocele detected during physical examination, grade 1, 2 or 3, c: Total of occasional, single treatment and daily use. Occasional use of over the counter pain killers not included, d: STD: Sexually transmitted diseases (clamydia, gonorrhea, or syphilis), e: Hormonal treatment and/or surgery, f: Answered yes to "Have you ever been hit, kicked or otherwise injured so it caused swelling or discolouring of the scrotum?", g: Varicocelectomy, operations due to hydrocele, testicular torsion or inguinal hernia. Orchidopexi not included, h: Free testosterone calculated as described by Vermeulen *et al.* 1999, i: Total sperm count > 39 mill. and Progressive motile spermatozoa > 32 % and Morphologically normale spermatozoa > 4%, j: sperm concentration <15 mill/ml.

Statistical analyses: ¤ Chi-square test, # Multiple linear regression analysis adjusted for co-variates comparing group A and group B. P-values <0.05 are indicated in bold.

Table 2: Genotype and allele distribution (percentages) of *FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G of the whole study population (total) and stratified into Subgroup A (without factors potentially affecting testicular function i.e. varicocele, treated cryptorchidism, previous testicular infections, previous minor scrotal injury or operations) and subgroup B with one or more such factors.

Polymorphism	Total	Subgroup A	Subgroup B	p-value*
***FSHB*-211G>T**	**N=2,006**	**N=1,409**	**N=597**	
Genotype				
GG	68.9	70.3	65.8	0.127
GT	28.6	27.5	31.2	
TT	2.5	2.3	3.0	
Allele				
G	83.2	84.0	81.4	**0.045**
T	16.8	16.0	18.6	
***FSHR*-29G>A**	**N=2,014**	**N=1,415**	**N=599**	
Genotype				
GG	54.1	53.5	55.4	0.723
GA	38.9	39.4	37.9	
AA	7.0	7.1	6.7	
Allele				
G	73.5	73.2	74.4	0.432
A	26.5	26.8	25.6	
***FSHR* 2039A>G**	**n=2,000**	**n=1,406**	**n=594**	
Genotype				
AA	29.3	29.9	27.9	0.143
AG	48.9	49.4	47.5	
GG	21.8	20.6	24.6	
Allele				
A	53.8	54.7	51.7	0.085
G	46.2	45.3	48.3	

* subgroup A vs. Subgroup B tested by Chi-square test.

P-values <0.05 are indicated in bold.

Table 3: Genotype and allele distribution (percentages) of *FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G stratified into < or > median levels of FSH, Inhibin B and testis size, as well as < or > than the WHO criteria for normal levels of total sperm count, in Subgroup A (without factors potentially affecting testicular function i.e. varicocele, treated cryptorchidism, previous testicular infections, previous minor scrotal injury or operations)

Polymorphism	FSH (IU/l)		p-value*	Inhibin B (pg/ml)		p-value*	Testis size (ml)		p-value*	Total sperm count		p-value*
	<2.48	**≥2.48**		**<171,9**	**≥171,9**		**<13.5**	**≥13.5**		**<39 mill.**	**≥39 mill.**	
***FSHB*-211G>T**												
Genotype												
GG	63.5	76.9	**<0.001**	67.2	73.3	**0.023**	67.3	73.0	**0.034**	72.6	69.7	0.735
GT	32.9	22.1		29.8	25.1		29.8	25.5		25.1	28.0	
TT	3.6	1.0		3.0	1.6		2.9	1.5		2.3	2.3	
Allele												
G	80.0	87.9	**<0.001**	82.1	85.8	**0.007**	82.2	85.5	**0.013**	85.1	83.7	0.500
T	20.0	12.1		17.9	14.2		17.8	14.2		14.9	16.3	
***FSHR*-29G>A**												
Genotype												
GG	54.3	52.6	0.348	51.7	55.0	0.215	50.8	55.3	0.239	44.6	54.8	**0.028**
GA	37.9	41.1		41.7	37.3		41.5	38.2		45.7	38.6	
AA	7.8	6.4		6.6	7.7		7.7	6.5		9.7	6.6	
Allele												
G	73.2	73.1	0.935	72.6	73.6	0.523	71.5	74.4	0.098	67.4	74.1	**0.008**
A	26.8	26.90		27.4	26.4		28.5	25.6		32.6	25.9	
***FSHR* 2039A>G**												
Genotype												
AA	31.0	29.2	0.738	29.7	30.6	0.05	27.4	31.9	0.079	25.9	30.5	0.378
AG	49.0	49.8		47.3	51.5		49.4	49.1		54.0	48.8	
GG	20.0	21.1		23.1	17.9		23.2	19.0		20.1	20.7	
Allele												
A	55.5	54.1	0.450	53.3	56.3	0.109	52.1	56.5	**0.024**	52.9	54.1	0.483
G	44.5	45.9		46.7	43.7		47.9	43.4		47.1	54.9	

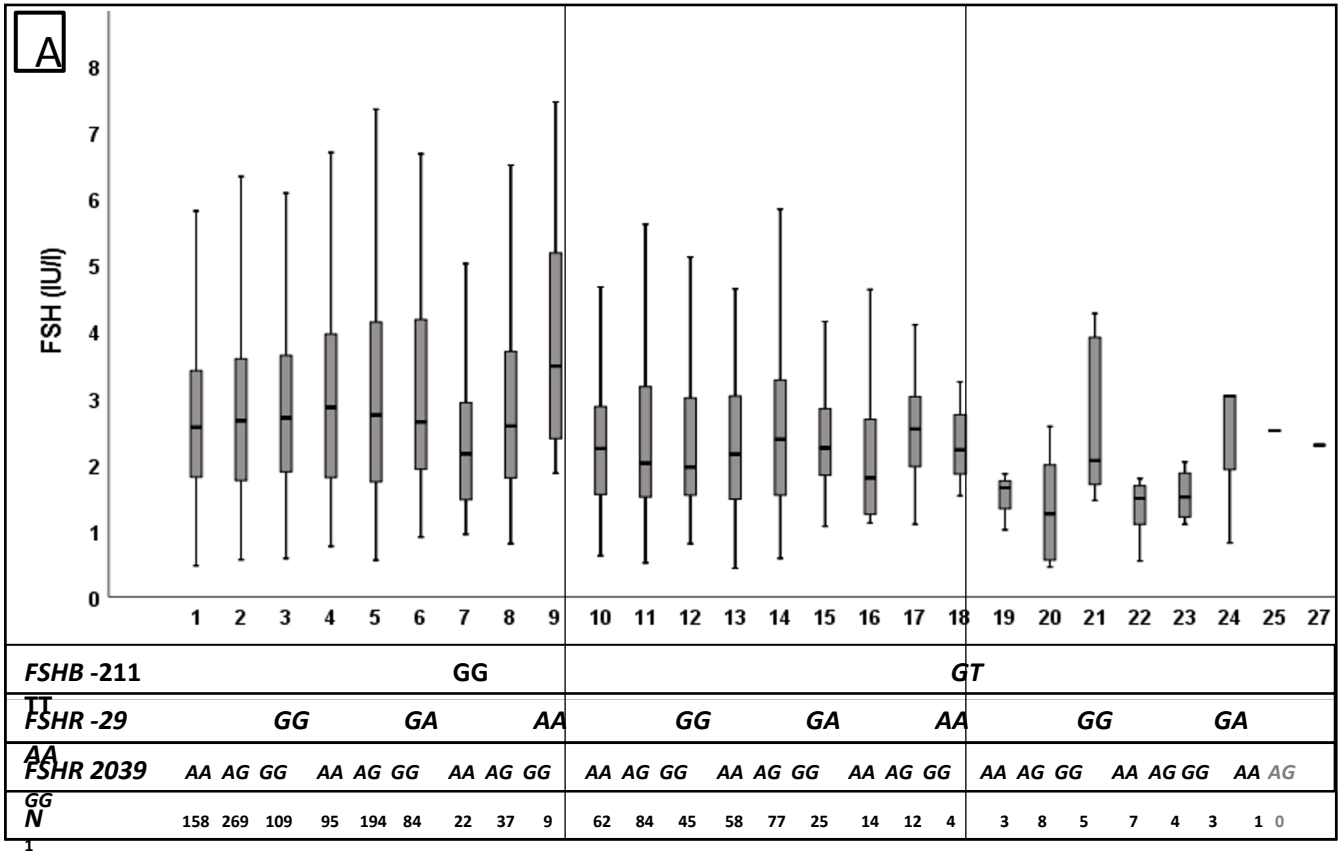
*Statistical difference between the two groups tested by Chi-square test. P-values <0.05 are indicated in bold.

Table 4: Semen parameters, testis size and hormone levels stratified according to *FSHB* -211G>T, *FSHR* -29G>A and *FSHR* 2039A>G in men without any known factors that could potentially affect the testicular function (N=1,491, subgroup A). Numbers are median values (min-max). Levels are shown unadjusted and stratified in eight groups according to the combination of the three SNPs described as major allele carriers vs. homozygote and heterozygote carriers (dominant model). Men with azoospermia are not included, but oligozoospermia <0.5 million/ml is shown as 0 million/ml.

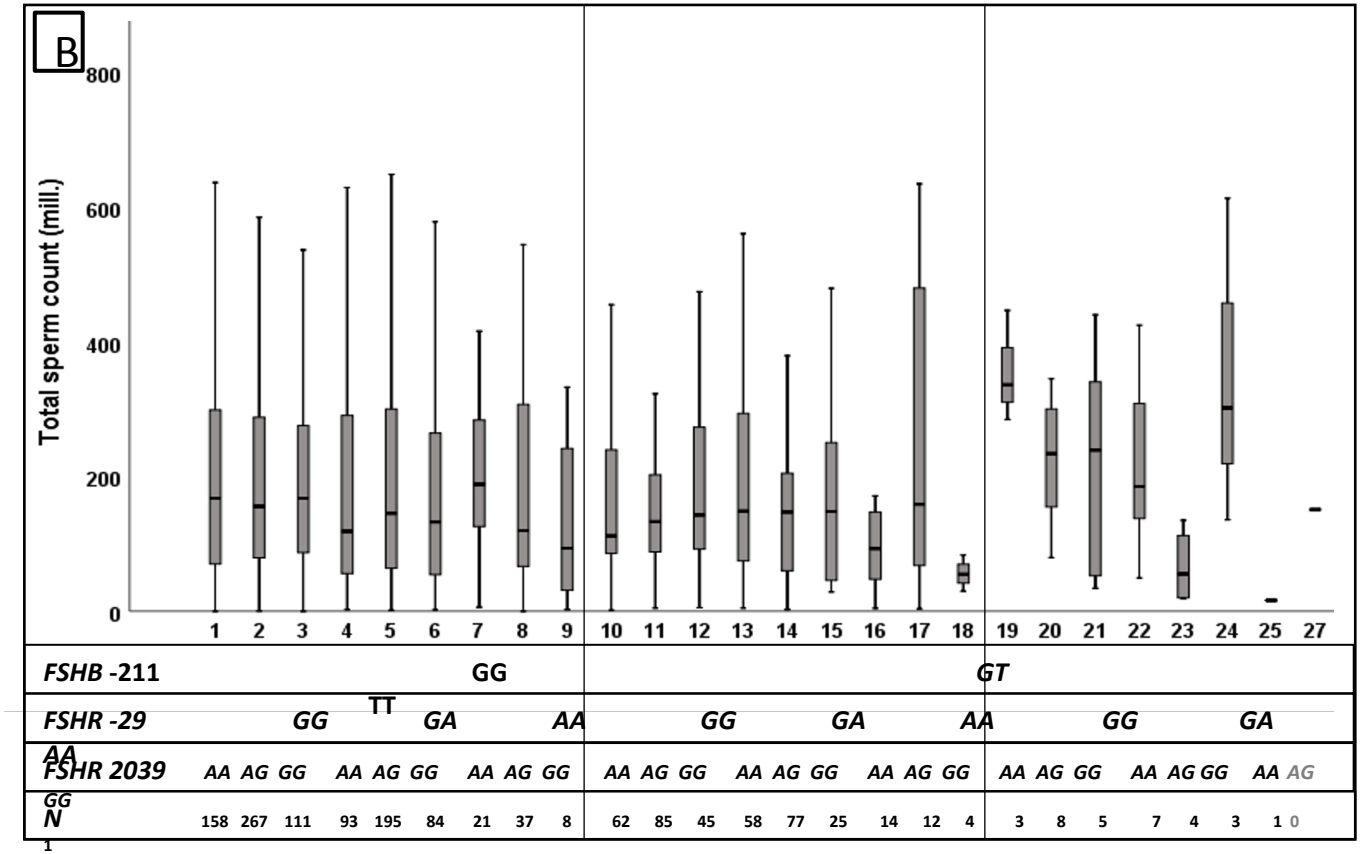
***FSHB* -211G>T**	**GG**				**GT/TT**			
***FSHR* -29G>A**	**GG**		**GA/AA**		**GG**		**GA/AA**	
***FSHR* 2039A>G**	**AA**	**AG/GG**	**AA**	**AG/GG**	**AA**	**AG/GG**	**AA**	**AG/GG**
	11.4%, N= 159	**27.3 %, N=382**	**8.4 %, N= 117**	**23.3 %, N= 326**	**4.6 %, N= 65**	**10.2 %, N= 143**	**5.7 %, N= 80**	**9.1%, N= 127**
Mean testis size, ultrasound (ml)	14.2 (7.5-25.0)	13.9 (7.0-31.8)	14.0 (7.4-23.4)	13.1 (4.6-28.9)	12.6 (5.9-23.9)	13.2 (4.7-21.8)	13.6 (5.2-25.0)	12.6 (5.0-23.3)
Total sperm count (mill)	168 (0-1326)	161 (0-1342)	136 (2-719)	139 (0-1151)	117 (1-802)	144 (5-844)	146 (4-805)	144 (3-817)
Total progressive sperm count (mill)	104 (0-862)	91 (0-895)	85 (0-534)	84 (0-686)	69 (0-388)	79 (0-549)	73 (2-561)	81 (1-575)
Total morphologically normal spermatozoa (mill)	13.2 (0.0-179.0)	10.1 (0.0-181.3)	9.6 (0.0-161.4)	8.7 (0.0-145.7)	8.1 (0.0-180.5)	9.1 (0.1-97.1)	8.2 (0.2-96.5)	7.3 (0.0-86.4)
FSH (IU/l)	2.6 (0.5-7.1)	2.7 (0.6-13.8)	2.6 (0.8-7.0)	2.7 (0.6-13.0)	2.2 (0.6-8.1)	2.0 (0.5-7.3)	2.0 (0.4-16.5)	2.3 (0.6-8.3)
Inhibin B (pg/ml)	177 (32-379)	174 (19-490)	171 (66-338)	172 (20-422)	167 (54-395)	169 (49-343)	170 (21-362)	162 (53-432)
Inhibin B/FSH	71 (7-643)	67 (3-579)	58 (12-380)	66 (2-384)	70 (10-560)	80 (12-624)	82 (1-593)	66 (8-426)
LH (IU/l)	3.2 (0.7-8.7)	3.3 (1.1-11.3)	3.6 (1.2-8.3)	3.3 (0.7-11.6)	3.5 (1.3-9.9)	3.6 (1.2-9.8)	3.3 (1.0-10.2)	3.6 (0.9-8.9)
Testosterone (nmol/l)	19.9 (4.7-54.0)	20.0 (8.5-64.1)	19.9 (10.5-56.1)	19.9 (6.8-52.8)	19.9 (10.3-39.6)	20.6 (4.1-44.3)	18.7 (9.1-42.0)	19.9 (7.1-44.5)
Testosterone/LH	6.5 (1.8-18.8)	6.1 (1.9-40.1)	5.9 (1.8-16.4)	5.8 (1.5-46.8)	6.2 (1.6-17.5)	5.7 (2.2-15.2)	5.5 (1.8-17.0)	5.7 (1.4-19.4)

Table 5: Overview of the effect estimates of the three FSH-related SNPs on markers of testicular function in the subgroup without any known factors that could potentially affect the testicular function (n=1,491). Multiple linear regression analyses were adjusted for co-variates and effect of the two other SNPs. P-values were based on models including the transformation of variables giving the best model fit (described in the statistical section). P-values <0.05 are indicated in bold.

	***FSHB* -211G>T**				***FSHR* -29G>A**				***FSHR* 2039A>G**			
	GG → GT		**GG →TT**		**GG → GA**		**GG → AA**		**AA → AG**		**AA → GG**	
	β (95% CI) ml	**p-value**	**β (95% CI) ml**	**p-value**	**β (95% CI) ml**	**p-value**	**β (95% CI) ml**	**p-value**	**β (95% CI) ml**	**p-value**	**β (95% CI) ml**	**p-value**
Mean testis size, ultrasound (ml)	-0.74 (-1.20;-0.28)	**0.002**	-1.35 (-2.74;0.04)	0.056	-0.38 (-0.81;0.04)	0.078	-0.73 (-1.53;0.08)	0.077	-0.23 (-0.70;0.24)	0.342	-0.76 (-1.34;-0.18)	**0.010**
	β (95% CI) %	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**
Total sperm count (mill)	-0.15 (-0.34;0.04)	0.126	0.09 (-0.48;0.66)	0.746	-0.17 (-0.34;-0.01)	0.069	-0.17 (-0.51;0.17)	0.326	-0.07 (-0.27;0.13)	0.500	0.05 (-0.19;0.30)	0.676
Total progressively sperm count (mill)	-0.17 (-0.35;0.01)	0.059	0.12 (-0.42;0.66)	0.666	-0.13 (-0.30;0.03)	0.119	-0.09 (-0.41;0.24)	0.600	-0.11 (-0.29;0.08)	0.267	0.04 (-0.19;0.27)	0.724
Total morphologically normal spermatozoa (mill)	-0.13 (-0.25;-0.00)	**0.043**	-0.07 (-0.42;0.29)	0.715	-0.04 (-0.15;0.08)	0.544	-0.09 (-0.31;0.12)	0.398	-0.08 (-0.20;0.05)	0.211	-0.01 (-0.17;0.14)	0.878
	β (95% CI) %	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**	**β (95% CI) %**	**p-value**
FSH (IU/l)	-0.17 (-0.23;-0.10)	**<0.001**	-0.52 (-0.71;-0.32)	**<0.001**	0.07 (0.01;0.13)	**0.030**	0.02 (-0.10;0.14)	0.762	0.05 (-0.02;0.11)	0.172	0.10 (0.01;0.18)	**0.026**
Inhibin B (pg/ml)	-0.04 (-0.08;0.01)	0.117	-0.09 (-0.23;0.05)	0.195	-0.04 (-0.08;0.01)	0.095	0.02 (-0.07;0.10)	0.692	0.00 (-0.04;0.05)	0.864	-0.04 (-0.10;0.02)	0.193
Inhibin B / FSH	0.13 (0.03;0.22)	**0.011**	0.43 (0.14;0.71)	**0.003**	-0.11 (-0.20;-0.01)	**0.023**	0.00 (-0.18;0.17)	0.987	-0.04 (-0.14;0.06)	0.411	-0.14 (-0.26;-0.01)	**0.032**
LH (IU/l)	0.04(-0.01.;0.09)	0.105	0.11 (-0.05;0.26)	0.172	0.04 (-0.01;0.09)	0.094	-0.02 (-0.11;0.07)	0.678	0.03 (-0.02;0.08)	0.253	0.04 (-0.03;0.10)	0.275
Testosterone (pmol/l)	-0.01 (-0.04;0.03)	0.791	-0.01(-0.12;0.09)	0.802	0.00 (-0.03;0.03)	0.996	-0.03 (-0.09;0.04)	0.448	0.00 (-0.04;0.04)	0.862	0.01 (-0.04;0.06)	0.640
Testosterone/LH	-0.05 (-0.10;0.00)	0.064	-0.12(-0.27;0.03)	0.114	-0.04 (-0.09;0.01)	0.086	-0.01 (-0.10;0.08)	0.897	-0.03 (-0.08;0.02)	0.297	-0.03 (-0.09;0.04)	0.438

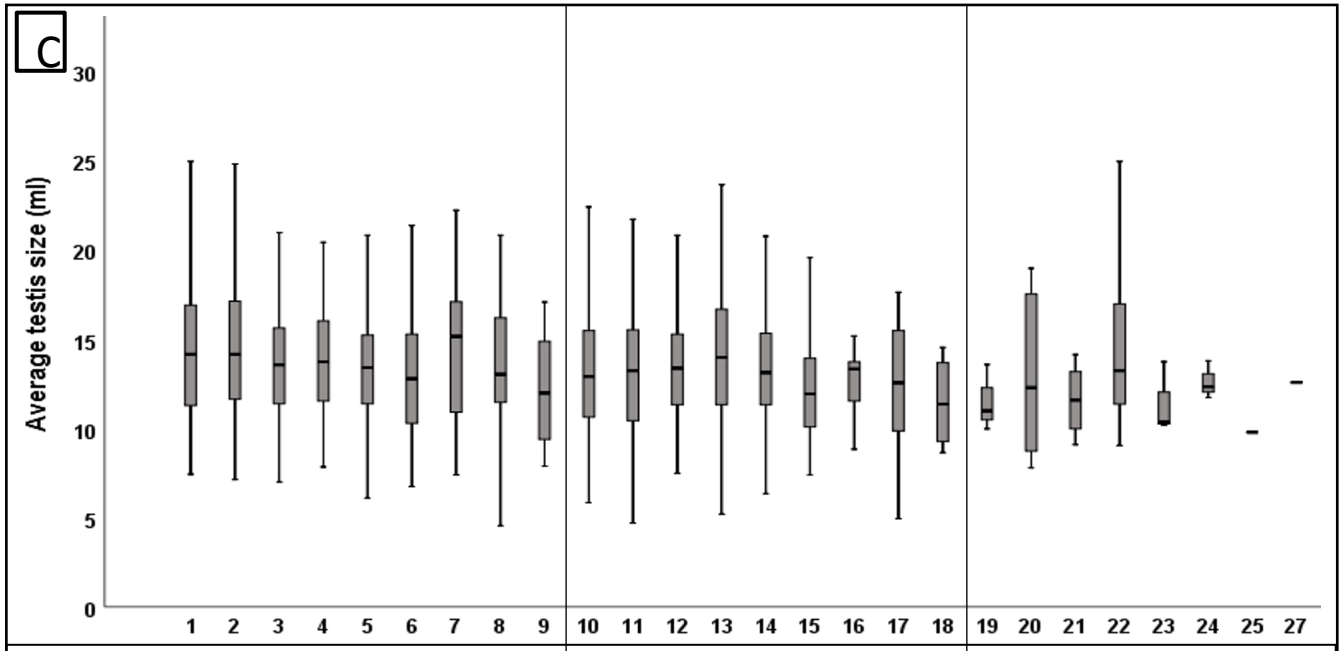


FSHB -211	GG									*GT*																	
FSHR -29	*GG*			*GA*			*AA*			*GG*			*GA*			*AA*			*GG*			*GA*					
FSHR 2039	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	
N	158	269	109	95	194	84	22	37	9	62	84	45	58	77	25	14	12	4	3	8	5	7	4	3	1	0	



FSHB -211										GG																	
FSHR -29				*GG*					*GA*					*AA*				*GG*				*GA*				*AA*	
FSHR 2039	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*
N	158	267	111	93	195	84	21	37	8	62	85	45	58	77	25	14	12	4	3	8	5	7	4	3	1	0	1

FSHB -211										GT																	
FSHR -29				*GG*					*GA*					*AA*				*GG*				*GA*				*AA*	
FSHR 2039	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*
N	62	85	45	58	77	25	14	12	4	3	8	5	7	4	3	1	0	1									



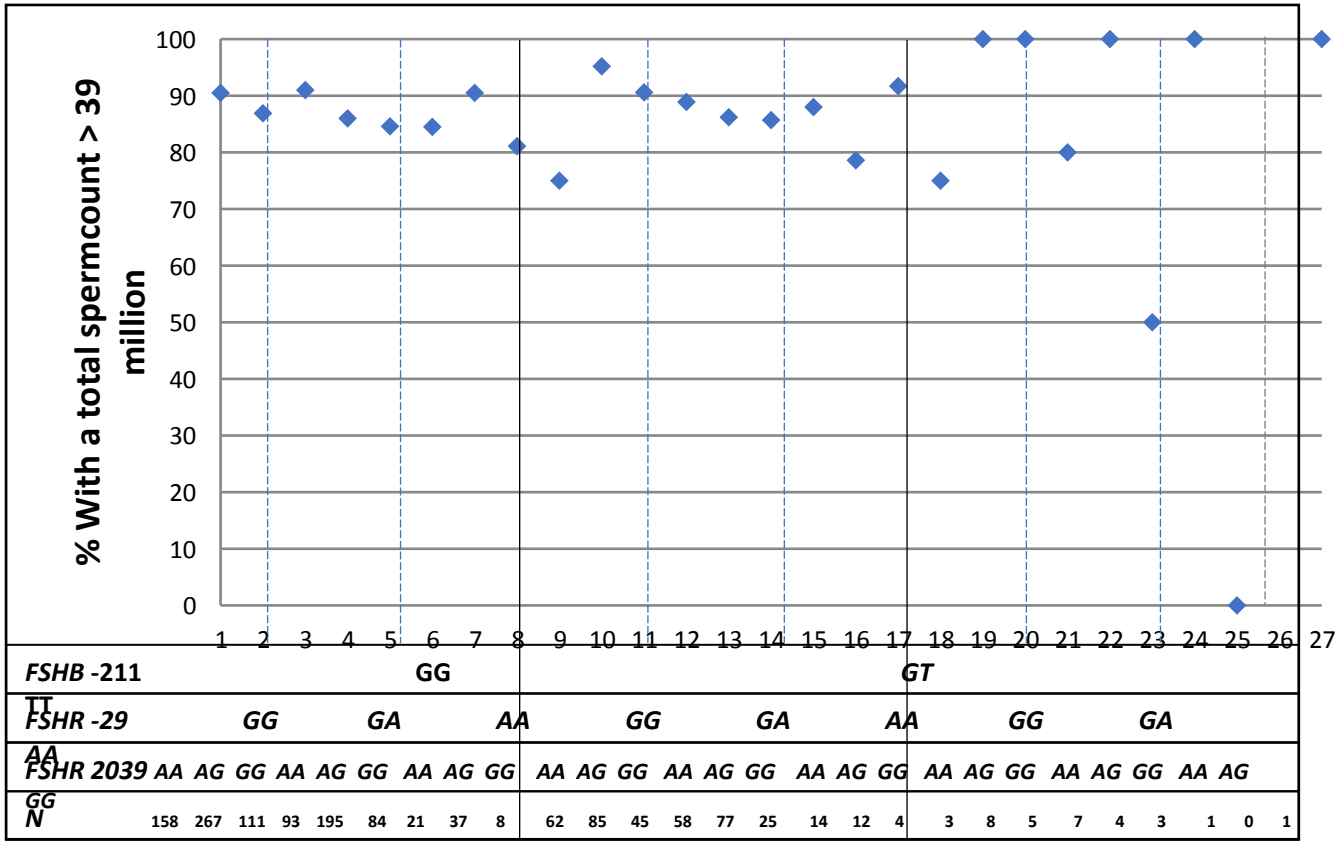
FSHB -211	GG									GT																	
FSHR -29	*GG*			*GA*			*AA*			*GG*			*GA*			*AA*			*GG*			*GA*					
FSHR 2039	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	*GG*	*AA*	*AG*	
N	148	254	106	87	186	82	20	35	9	58	78	42	57	74	25	13	12	4	3	6	5	7	3	3	1	0	

TT

AA

GG

1


% With a total spermcount > 39 million
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
FSHB -211
GG
GT
TT
FSHR -29
GG
GA
AA
GG
GA
AA
GG
GA
AA
FSHR 2039
AA AG GG AA AG GG AA AG GG AA AG GG AA AG GG AA AG GG AA AG GG AA AG GG AA AG GG AA AG
GG
N
158 267 111 93 195 84 21 37 8 62 85 45 58 77 25 14 12 4 3 8 5 7 4 3 1 0 1