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The effect of a specific histidine-rich glycoprotein polymorphism on male infertility and semen parameters




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Abstract In women, there is evidence that a single nucleotide polymorphism (SNP) in the *histidine-rich glycoprotein (HRG)* named *HRG C633T* is relevant for a number of fertility outcomes including recurrent miscarriage, ovarian response and pregnancy outcome after IVF. This case-control study was designed to investigate whether the *HRG C633T* SNP is important for male infertility and pregnancy rate following IVF. Cases were 139 infertile couples and controls were 196 pregnant couples. The 335 couples all contributed with one blood sample per partner. Genomic DNA was extracted and genotyping was performed using a TaqMan® SNP Genotyping Assay. Information on pregnancy rate and semen parameters was derived from medical records. Infertile couples in which the male partner was a homozygous carrier of the *HRG C633T* SNP had significantly lower ($P < 0.01$) pregnancy rate following IVF in comparison with couples where the male partner was a heterozygous *HRG C633T* SNP carrier. Male homozygous *HRG C633T* SNP carriers had overall lower total sperm count, sperm concentration, motility score and yield after preparation. In conclusion, once infertility is established the *HRG C633T* SNP seems to be important for male infertility and pregnancy rate following IVF. 

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KEYWORDS: angiogenesis, histidine-rich glycoprotein, infertile couples, male infertility, semen parameters, single nucleotide polymorphism

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Introduction

Infertility affects approximately 15% of couples around the world, and the underlying causes can be grouped into male factors, female factors and unexplained infertility (Kamath and Bhattacharya, 2012). Among these, male infertility, alone or combined with female factors, accounts for up to 50% of cases (Esteves et al., 2012). Many infertile couples are helped by IVF, which remains the treatment of choice in long-standing, unresolved infertility (Kamath and Bhattacharya, 2012).

Many factors, such as patient age, body mass index (BMI), smoking and previous surgery (Andersen et al., 2015; Esteves et al., 2012; Pinborg et al., 2011; Waylen et al., 2009), affect a couple's response to assisted reproductive treatment. Genetic make-up may also influence the response and outcome of assisted reproductive treatment (Venkatesh et al., 2014). It is estimated that 10–15% of spermatogenic impairment is explained by genetic defects (Ferlin and Foresta, 2014), and furthermore, association studies propose gene polymorphisms to affect spermatogenesis, which in turn may lead to oligozoospermia- or azoospermia-induced male infertility (Chen et al., 2015; Ge et al., 2015; Plaseski et al., 2012; Sato et al., 2015).

We have previously shown that a genetic variant of the *histidine-rich glycoprotein* (HRG) gene may be relevant for pregnancy success rate following IVF (Nordqvist et al., 2011b) but also for recurrent miscarriage (Lindgren et al., 2013). This single nucleotide polymorphism (SNP) is denoted HRG C633T and has either a cytosine (C) or a thymine (T) at position 633 in the gene. The result of the HRG C633T SNP is a missense variation where a cytosine codes for a proline amino acid and a thymine will instead code for a serine at position 186 in the protein. The serine allows for a glycosylation at position 184 in the protein. The HRG C633T SNP is relatively common, with allele frequencies of 67% and 33% for C and T, respectively, in a mixed European population [http://www.ensembl.org/Homo_sapiens, rs9898]. HRG is a multi-domain protein that is synthesized by the liver and is present at high levels in plasma, where it seems to function as an adaptor protein. HRG is involved in coagulation, angiogenesis and the immune system (Jones et al., 2005; Poon et al., 2010; Wakabayashi, 2013), all of which are processes important for establishment of a pregnancy (Bazer et al., 2010; Thornton and Douglas, 2010). In addition, HRG interacts with thrombospondin, plasmin and plasminogen, IgG, fibrinogen, heparin, vascular endothelial growth factor (VEGF) and various members of the fibroblast growth factor (FGF) family (Gorgani et al., 1997; Jones et al., 2004; Lerch et al., 1988; Simantov et al., 2001; Vu et al., 2011; Wakabayashi and Koide, 2011; Wake et al., 2009). Again, many of these ligands have been associated with infertility (Cotton et al., 2008; Ebisch et al., 2008; Lash et al., 2012).

We have previously demonstrated that women with unexplained infertility who were homozygous T/T-carriers of the SNP (HRG 633T SNP) had lower than expected pregnancy rates following IVF. Correspondingly in another study, the homozygous HRG 633T SNP carriers had fewer oocytes retrieved despite higher levels of administered FSH, even though relevant demographic factors such as age, antral follicle count and anti-Müllerian hormone (AMH) levels were similar between groups (Nordqvist et al., 2015). However, it has not been

established whether the SNP is more common in infertile couples than in pregnant control couples, and furthermore, no studies have investigated this SNP in relation to male infertility and semen parameters. Thus, the aim of this study was to investigate whether male HRG C633T genotype is associated with pregnancy rate following IVF and semen parameter values in infertile couples. A secondary aim was to explore whether the HRG C633T SNP distribution is different between infertile couples and a control population of pregnant couples without infertility history.

Materials and methods

Ethics

The study was approved by the Regional Ethics Review Board in Stockholm (diary numbers: 2007/3–31/3, 2007/1559–32, approval 7 March 2007; 2010/0180–32, amendment approval 12 February 2010). All couples met with a healthcare professional and received written and verbal information about the study and gave written informed consent. Participation was voluntary, not reimbursed and did not affect treatment.

Study population

Heterosexual couples visiting an IVF clinic (Fertilitetscentrum, Stockholm) between 1 March 2010 and 6 February 2012 were asked to participate in the study. The answering frequency is not known. Couples were fluent in Swedish or English and contributed with one blood sample per person. Blood samples were collected on the day of ovum pick-up. All couples had previously undergone assessment for cause of infertility.

Infertile women were screened for anovulation, including polycystic ovarian syndrome (PCOS), according to Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group, 2004), thyroid dysfunction, tubal factors, endometriosis and uterine anomalies. Basal FSH levels or AMH levels were tested, and FSH levels below 13 IU/l or AMH levels above 0.7 ng/ml were considered normal.

Infertile men were screened for previous infections (in testis, epididymis and prostate), previous surgery (inguinal hernia, genital tract or urinary tract) and medical history. Male BMI was not available. Semen analyses were routinely performed by embryologists and included the number of and motility of spermatozoa. Normally, only one semen sample is analysed during a fertility investigation at this clinic, but if the results are deviant the test is repeated. Sperm counts below the World Health Organization guideline levels (Tocci and Lucchini, 2010) were classified as male infertility. A motility score was used, ranging from zero to three, where zero defines immobile spermatozoa and three corresponds to the highest progressive motility.

Couples were included regardless of infertility diagnosis even if the infertility was unexplained or had multiple causes. Overall, 155 couples consented to participate. Among these, genotyping was completed in 139 couples. The couple's infertility diagnoses are listed in [Table 1](#).

Obstetric and medical history of the infertile couples is displayed in [Supplementary Table S1](#). About one-fifth of couples

Table 1 Treatment and causes of infertility, in the total study population and according to male *HRG* C633T SNP.

	<i>Total sample</i>	<i>Male HRG C633T SNP</i>		
	<i>n = 139</i>	<i>C633 n = 72</i>	<i>C633T n = 51</i>	<i>633T n = 16</i>
Cause of infertility				
Anovulatory	15 (10.8)	8 (11.1)	5 (9.8)	2 (12.5)
Tubal factor	10 (7.2)	7 (9.7)	3 (5.9)	0
Endometriosis	7 (5.0)	3 (4.2)	4 (7.8)	0
Unexplained	68 (48.9)	35 (48.6)	25 (49.0)	8 (50.0)
Male infertility	26 (18.7)	9 (12.5)	12 (23.5)	5 (31.3)
Multiple diagnoses ^a	10 (7.2)	7 (9.7)	2 (3.9)	1 (6.3)
Other	3 (2.2)	3 (4.2)	0	0
Male infertility as single or combined factor	35 (25.2)	15 (20.8)	14 (27.5)	6 (37.5)
Infertility duration (years)	2.5 (2.0–3.5)	2.5 (2.0–3.25)	2.5 (2.0–3.5)	2.5 (1.6–3.0)
Total treatments ^b	1.0 (1.0–2.0)	1.0 (1.0–2.0)	1.0 (1.0–1.0) ^c	1.5 (1.0–2.75)
IVF	96 (69.1)	50 (69.4)	37 (72.5)	9 (56.3)
ICSI	36 (25.9)	20 (27.8)	11 (21.6)	5 (31.3)
Combined IVF/ICSI	7 (5.0)	2 (2.8)	3 (5.9)	2 (12.5)
Embryos from IVF transferred	81 (58.3)	40 (55.6)	34 (66.7)	7 (43.8)
Embryos from ICSI transferred	38 (27.3)	20 (27.8)	13 (25.5)	5 (31.3)
No embryo transfer	20 (14.4)	12 (16.7)	4 (7.8)	4 (25.0)

Data are presented as median (interquartile range) or number (%).

IVF = conventional IVF; ICSI = intracytoplasmic sperm injection.

^aIncluding both male/female causes and couples with only multiple female causes and no male cause.

^bTotal treatments including the current treatment.

^cSignificantly lower than homozygous C633 and 633T, $P < 0.01$, respectively, Kruskal–Wallis and post hoc Mann–Whitney U -test.

had been pregnant before (22.3%) and almost one-fifth (18.7%) had experienced at least one miscarriage. Only two out of the 139 couples (1.4%) had a child together. The frequencies of previous pregnancies and miscarriages were about the same among men and women but a higher proportion of men had a child from a previous or current relationship (14.4% versus 5.0%, $P < 0.05$). Hypothyroidism was reported in eight women (5.8%), diabetes (type 1 or type 2 not specified) in two women and one man, and thrombophilia in two women. **Supplementary Table S2** displays previous surgery and infections of the infertile men. Surgery for inguinal hernia, testicular disorders and varicocele was reported by 18 men. Infections of epididymis and prostate were reported by six men, and eight men had a history of chlamydia. Semen test results were available for all but two men ($n = 137$).

The control group consisted of heterosexual pregnant couples without previous infertility, who were approached in gestational week 16–19, in conjunction with the routine ultrasound scan at Uppsala University Hospital, between February 2012 and December 2012. Control couples were 18 years of age or older, had conceived spontaneously and were able to understand Swedish or English. Each couple contributed with one blood sample per person on the day of the ultrasound scan. In total, 196 control couples were included and genotyping was successful in all. The women's medical records were reviewed for data on age, weight, height and smoking/snuff habits from their admission to the maternity centre in the first trimester. Female demographic data such as BMI and smoking/snuff habits were not entirely comparable to the infertile couples because the latter group was not pregnant

at the time of enrolment. Male BMI and smoking/snuff habits were not available.

IVF treatment

The IVF treatment protocols at the Fertilitetscentrum Stockholm have previously been described in detail (Nordqvist et al., 2015). In brief, starting dosages of FSH were determined individually based on anticipated ovarian response and, whenever possible, on previous treatment results. Ultrasound evaluation began on stimulation day 5–10 and was repeated if necessary. Dosages could be changed at the ultrasound depending on results. Women underwent either a gonadotrophin-releasing hormone (GnRH) agonist protocol ($n = 108$) or GnRH antagonist protocol ($n = 31$). The women received recombinant FSH ($n = 114$) or human menopausal gonadotrophins (HMG) ($n = 25$). Only ejaculated partner spermatozoa were used. Intracytoplasmic sperm injection (ICSI) was used if the number of prepared spermatozoa using PureSperm® (Nidacon, Sweden) was ≤ 1 million or in cases with previous low fertilization ($<50\%$). Conventional IVF ($n = 95$), ICSI ($n = 38$) or a combination of both ($n = 5$) were used. Embryos were assessed according to standardized clinical morphological protocols (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), and transfer was not performed if there were signs of abnormal growth. Single-embryo transfer was performed in most cases ($n = 110$) and double-embryo transfer was performed if the risk of having twins was low ($n = 9$).

Among infertile couples, 51 (36.7%) became pregnant after treatment, 68 (48.9%) did not become pregnant, and 20 (14.4%) couples had no embryo transfer for reasons mostly related to pathological development of the embryo or no oocytes being fertilized ($n = 16$, 80.0%). Four couples did not have an embryo transfer due to a risk of ovarian hyperstimulation syndrome ($n = 2$), for social reasons ($n = 1$) and due to technical difficulties with the transfer ($n = 1$).

Blood sampling and DNA extraction

Venous blood samples were collected in EDTA-containing tubes on the day of ovum pick-up for the infertile couples and at the ultrasound scan for the controls. All samples from both groups were handled equally. After a centrifugation step (1500g for 10 min), buffy coat was transferred to new tubes and stored at -20°C until transferred weekly to -70°C storage. The buffy coat from 16 of the infertile couples was discarded by accident and therefore it was not possible to perform the subsequent analyses on these. Genomic DNA was extracted from the buffy coat using QIAamp DNA Blood Mini Kits (Qiagen, Venlo, The Netherlands).

Genotyping

Genotype was determined after embryo transfer. The samples were genotyped for the HRG C633T SNP (rs9898), using the TaqMan® SNP Genotyping Assay and run with the StepOnePlus real-time PCR system (Life Technologies Inc.). Briefly, polymerase chain reactions were performed in 96-well plates in a total volume of 25 μl for each reaction. Each reaction consisted of 1 \times TaqMan Universal PCR Master Mix (PCR buffer, ROX passive reference dye, dNTPs and AmpliTaq Gold DNA Polymerase), 1 \times SNP Genotyping Assay (sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest, i.e. HRG exon 5, TaqMan MGB probes labelled with VIC dye to detect allele 1 sequence and with FAM to detect allele 2 sequence) and 10 ng of genomic DNA. Cycling conditions were initiated for 10 min at 95°C followed by 40 cycles of 15 s at 92°C and 1 min at 60°C . Real-time fluorescence detection was performed. StepOne™ Software (Life Technologies Inc.) was used to plot fluorescence (R_n) values based on the signals from each well. The plotted fluorescence signals indicated which alleles were present in each sample.

Statistics

Couples in whom embryo transfer was not performed due to pathological development of the embryo or no oocytes being fertilized ($n = 16$, 80.0%) were included in the non-pregnant group. The four couples who did not have an embryo transfer, for other reasons, were excluded.

The number of homozygous and heterozygous carriers of the HRG C633T SNP was in Hardy-Weinberg equilibrium ($X^2 = 0.114$). For skewed data, the Kruskal-Wallis test was employed, followed by post hoc Mann-Whitney U -test. Categorical data were analysed with the Pearson chi-squared test or

Fisher's exact test. All statistical tests were two-sided and P -values of <0.05 were considered significant. The Statistical Package for the Social Sciences version 20.0 was used for all statistical analysis (IBM Corp., USA).

Results

Background characteristics and frequency of the HRG C633T SNP

In total, 139 infertile and 196 pregnant couples were included in the study. The infertile women and men were older than the corresponding controls (women; 34 (31–36) versus 31 (27–34) years; $P < 0.001$ and men; 36 (33–39) versus 32 (29–35) years; $P < 0.001$). Across the HRG C633T SNP no differences in age, BMI, smoking or snuff usage were noted (Supplementary Table S3). The distribution of the HRG C633T SNP did not differ between infertile women and men and their respective controls (Table 2). Furthermore, no difference in frequency of T-alleles between infertile and control couples was noted (Figure 1). However, men diagnosed with male factor infertility (as single factor) were more often homozygous HRG 633T SNP carriers than men in the pregnant control group, but this was only borderline significant (HRG 633T SNP; male factor infertility 19.2% versus control men 7.1%; $P = 0.05$).

Table 2 Frequency of the HRG C633T SNP in infertile men and women, and respective controls.

	Men		Women	
	Infertile $n = 139$	Control $n = 196$	Infertile $n = 139$	Control $n = 196$
HRG C633T				
C633, n (%)	69 (49.6)	97 (49.5)	72 (51.8)	108 (55.1)
C633T, n (%)	57 (41.0)	78 (39.8)	51 (36.7)	74 (37.8)
633T, n (%)	13 (9.4)	21 (10.7)	16 (11.5)	14 (7.1)

No significant differences in genotype frequencies noted, Pearson chi-squared test.

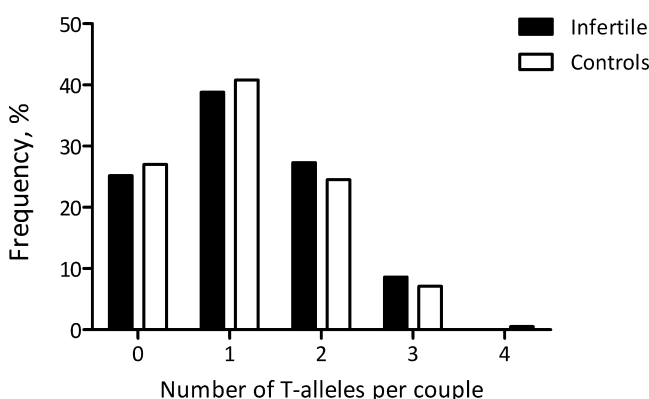


Figure 1 T-allele frequency of the HRG 633T SNP in infertile and pregnant control couples. No difference in frequency of T-alleles between infertile and control couples was noted, Pearson chi-squared test.

IVF treatment and pregnancy rate

Detailed information on the infertile women's IVF treatment parameters in relation to the different *HRG* C633T SNPs has been presented previously (Nordqvist et al., 2015). Briefly, no differences were found except that women who were homozygous *HRG* 633T SNP carriers needed significantly higher FSH dosages during treatment than the others (Nordqvist et al., 2015).

Among the infertile men no differences in infertility diagnoses, treatment type or number of embryo transfers were found across the homozygous and heterozygous carriers of the *HRG* C633T SNP (Table 1). However, the male heterozygous *HRG* C633T SNP carriers had undergone fewer previous IVF/ICSI treatments than the other groups, $P < 0.01$ (Table 1).

The *HRG* C633T SNP was not associated with pregnancy rate in the infertile women; however, in infertile men the *HRG* C633T SNP had an impact on the pregnancy rate of the current treatment cycle (Table 3). Most successful were couples with male heterozygosity where 52.0% achieved pregnancy, whereas only two couples (14.3%) became pregnant when the male partners were homozygous carriers of the *HRG* 633T SNP, $P < 0.01$.

These results remained even when couples with no embryo transfer were excluded from the analysis (data not shown).

Figure 2 shows the pregnancy rate in relation to the frequency of T-alleles in the infertile couples. Most successful were couples with one or two T-alleles, which corresponds to one or two heterozygous *HRG* 633T SNP carriers or only one homozygous *HRG* 633T SNP carrier in the couple, where 48.1% ($n = 26$) and 42.1% ($n = 16$) became pregnant, respectively (Figure 2A). Couples carrying no or three T-alleles in the *HRG* C633T SNP had a low pregnancy rate; only 20% ($n = 7$) of couples with no T-alleles and 16.7% ($n = 2$) of couples with three T-alleles became pregnant. Figure 2B displays pregnancy rate in the couples in which either partner was homozygous for the T-allele. While the numbers were too low for statistical comparisons it is noted that couples in which the woman was a homozygous *HRG* 633T SNP carrier were most successful. When two T-alleles were present in the couple and the woman was homozygous, 37.5% ($n = 3$) became pregnant compared with 22.2% ($n = 2$) if the man was carrying the homozygosity. When three T-alleles were carried by the couple, 40% ($n = 2$) became pregnant if the woman was homozygous and none if the man was homozygous.

Table 3 Treatment outcome according to *HRG* C633T SNP.

	Men, $n = 135^a$		Women, $n = 135^a$	
	Pregnant	Not pregnant ^b	Pregnant	Not pregnant ^b
In total, n (%)	51 (37.8)	84 (62.2)	51 (37.8)	84 (62.2)
<i>HRG</i> C633T, n (%)				
C633	23 (32.4)	48 (67.6)	22 (33.3)	44 (66.7)
C633T	26 (52.0)	24 (48.0)	24 (42.9)	32 (57.1)
633T	2 (14.3)	12 (85.7)**	5 (38.5)	8 (61.5)

** $P < 0.01$, Pearson chi-squared test.

^aFour patients are excluded due to no embryo transfer for reasons other than pathological development of the embryo or no oocytes being fertilized.

^bPatients with no embryo transfer due to pathological development of the embryo or no oocytes being fertilized are included in the non-pregnant group ($n = 16$).

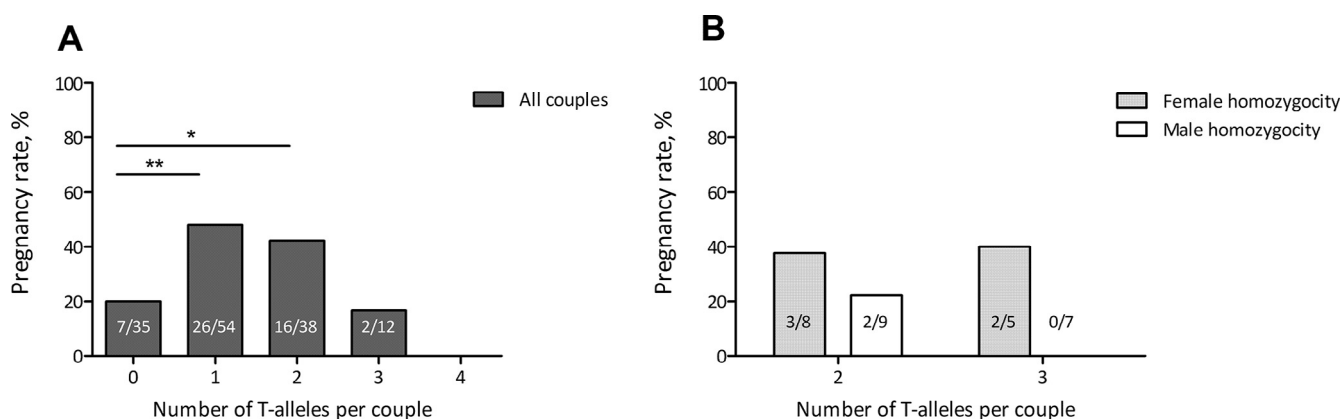


Figure 2 Pregnancy rate following IVF according to the frequency of T-alleles (*HRG* 633T SNP) in the infertile couples. Bars represent the percentage of pregnant couples according to the number of T-alleles within the couple. The total number of couples is given within each bar. (A) All pregnant couples. No couples had four T-alleles (homozygous 633T/633T), hence this bar is not shown. (B) Couples with one homozygous *HRG* 633T SNP carrier; either the woman (grey) or the man (white). Due to the small numbers no statistical analyses were performed. * $P < 0.05$, ** $P < 0.01$, Pearson chi-squared test.

Table 4 Semen parameters of 137 infertile men, according to male *HRG* C633T.

	<i>HRG</i> C633T					
	C633, n = 72		C633T, n = 49		633T, n = 16	
	n	Median (range)	n	Median (range)	n	Median (range)
Ejaculate volume (ml)	72	2.5 (2.0–4.0)	49	3.1 (2.6–4.0) ^a	16	2.5 (1.6–3.5)
Total sperm count (10 ⁶)	72	131 (57–247)	49	180 (63–274)	16	81 (38–106) ^{a,b}
Sperm concentration (×10 ⁶ /ml)	72	52.0 (29–85)	49	52 (23–85)	16	28 (14–50) ^a
Progressive motility (%)	72	59 (45–71)	48	62 (44–70)	15	65 (40–70)
Motility score (0–3) ^e	64	2.3 (2.2–2.4)	38	2.3 (2.2–2.4)	15	2.2 (2.0–2.3) ^{a,b}
Yield after preparation (%)	72	10.0 (2.5–20.0)	49	7.5 (1.2–20.5)	16	2.5 (0.6–10.1) ^a

^aSignificantly different from homozygous C633, $P < 0.05$ – 0.01 , Kruskal–Wallis and post hoc Mann–Whitney U -test.

^bSignificantly different from heterozygous C633T, $P < 0.05$ – 0.01 , Kruskal–Wallis and post hoc Mann–Whitney U -test.

Semen profile

The semen profile of 137 infertile men, according to the male *HRG* C633T SNP, is displayed in **Table 4**. Male homozygous *HRG* 633T SNP carriers had lower total sperm count and motility score compared with both homozygous *HRG* C633 SNP carriers and heterozygous *HRG* C633T SNP carriers. Moreover, homozygous *HRG* 633T SNP carriers had lower sperm concentration and yield after preparation compared with homozygous *HRG* C633 SNP carriers (**Table 4**). A sensitivity analysis, excluding all men with male factor infertility, rendered similar results, except for yield after preparation, which no longer remained significantly different across the *HRG* C633T SNP (data not shown).

Discussion

In women, the *HRG* C633T SNP has previously been shown to be relevant for a number of fertility outcomes (Lindgren et al., 2013; Nordqvist et al., 2011a), but its role in infertile men, and infertile couples, has until now not been investigated.

Our findings suggest that the *HRG* C633T SNP is equally distributed in infertile and pregnant control couples. This finding is not unexpected as infertility has many causes and is not considered a Mendelian inherited disease. However, once infertility is established the *HRG* C633T SNP seems to play a role; couples with male homozygous carriers of the *HRG* 633T SNP had a lower chance of a successful treatment, and male homozygous *HRG* 633T SNP carriers had the overall lowest semen parameter values.

When analysing the infertile couples, two important findings in regard to the expression of the *HRG* C633T SNP were revealed. First, couples carrying one or two T-alleles had higher pregnancy rates in comparison with couples with no T-allele present. This implies that the T-allele is needed in at least one copy in the couple for a higher chance of pregnancy. Secondly, in couples with more than two copies, i.e. with at least one partner being homozygous for the T-allele, pregnancy rates declined to 16.7%. As previously stated, this was especially relevant in couples with male homozygous carriers of the *HRG* 633T SNP, where pregnancy rates were 0% or 22%, depending on whether the female partner had a T-allele or not. Hence,

male *HRG* C633T SNP heterozygosity was superior in terms of IVF treatment outcome and was also associated with the lowest number of total treatments compared with the homozygous carriers. This is in line with the theory of heterozygote advantage in which heterozygous carriers present a selective advantage in viability and reproductive fitness over homozygotes in natural populations (Hansson and Westerberg, 2002). This theory has mostly been associated with disease resistance but is also described in relation to female fecundity. Laanpere et al. (2011) investigated infertile women undergoing IVF treatment and the importance of several different SNPs in the folate pathway. They reported positive associations between heterozygous carriers and different outcome parameters such as good-quality embryos and increased chance of pregnancy (Laanpere et al., 2011). Elenis et al. (2014) found that fewer women who were heterozygous carriers of another SNP in the *HRG* gene (A1042G) had recurrent miscarriage.

There are several reasons why the *HRG* C633T SNP may confer heterozygous advantage. First, the predominant/native HRG protein contains a proline at amino acid 186 (corresponding to the *HRG* C633 SNP). Proline is thought, due to its unique cyclical formation with a secondary amine, to be important for the protein structure and contributes to exceptional conformational rigidity. The variant protein that has a serine at amino acid 186 (corresponding to the *HRG* 633T SNP) allows for a glycosylation at position 184 in the protein (Jones et al., 2005; Kassar et al., 2014). In between the position in the protein where the amino acid shift might occur and the extra glycosylation an inter-domain disulphide bridge is found (amino acid 185). With the proline186serine shift and the interference of the glycosylation with the disulphide bridge nearby, HRG could be less stable or its function could be altered. If the variant HRG protein possesses functions somewhat different from the predominant/native protein, the heterozygous genotype could benefit from having both. Secondly, the disulphide bridge connects to a region in the protein known to have angiogenesis-regulating properties. It has been shown that a fragment from this region can, when released from the protein, exert an anti-angiogenic effect on endothelial cells via different mechanisms (Dixelius et al., 2006; Hennis et al., 1995; Kassar et al., 2014; Lee et al., 2006). HRG in its predominant form can inhibit both VEGF and FGF-mediated angiogenesis of endothelial cells *in vitro* (Olsson et al., 2004) but in the variant of the HRG protein where an amino acid

shift has occurred in position 186 the stability of the disulphide bridge might be altered and thereby disable the proteolytic release of the anti-angiogenic fragment. Finally, HRG is also known to have an indirect pro-angiogenic effect via the binding of thrombospondins (TSP) (Simantov et al., 2001). TSP exert their anti-angiogenic effect through binding of the CD36-receptor on endothelial cells and by inhibiting the response of a number of different growth factors important for angiogenesis such as FGF and VEGF (Febbraio et al., 2001). Indeed, a proteome analysis has located TSP1 and TSP2 to the testis, prostate and seminal vesicles of men (Wilhelm et al., 2014). HRG contains two CD36 homology domains and one of them is located around the polymorphic area (amino acid 155–213), that binds to TSP with high affinity, thereby blocking its anti-angiogenic activity (Jones et al., 2005; Simantov et al., 2005). The pro- and anti-angiogenic effects mentioned above could be lacking in the less common variant of the protein and so possibly contribute to the lower pregnancy rates seen in couples with male homozygous carriers of the *HRG* 633T SNP.

Male homozygous *HRG* 633T SNP carriers had overall lower semen test results. Furthermore, when men with male infertility were excluded the total sperm count, sperm concentration and motility score remained the lowest among carriers of the homozygous *HRG* 633T SNP. We have recently described that HRG exists in the female reproductive tract and also in human blastocysts (Nordqvist et al., 2010), but the presence and distribution of HRG in the male genital tract is not as well investigated. However, by mass spectrometry the HRG protein has been detected in the testis, prostate and seminal vesicles of the human male genital tract (Wilhelm et al., 2014), where it potentially could influence spermatogenesis. Normal spermatogenesis requires crosstalk between somatic and germ cells, relevant endocrine signalling (Singh and Jaiswal, 2011) and adequate blood flow (Ebisch et al., 2008). Vascular changes have been reported in testicular biopsies from infertile men (Cummins et al., 1994; Nasah and Cox, 1978), and potentially for the reasons given above, HRG may act as a regulator of angiogenesis during spermatogenesis and maturation of spermatozoa. It is known that transcripts and proteins important for early embryogenesis accompany the male DNA and are introduced to the oocyte through the spermatozoon (Jodar et al., 2016). It is possible that this set of transcripts and proteins can be altered by the milieu in which the spermatozoa are produced. The effect of this could be reflected in the characteristics measured in the sperm analysis but could also go unnoticed in couples without a male factor infertility. HRG also influences vascular endothelial growth factor (VEGF), another key player in angiogenesis, which is expressed by both Leydig and Sertoli cells in prostate vesicles, and in seminal vesicles (Olsson et al., 2004; Ergun et al., 1997). Importantly, VEGF is present in high levels in seminal plasma (Brown et al., 1995). Obermair et al. (1999) found the concentration of VEGF in seminal plasma to be the strongest predictor of the chance of pregnancy in IVF treatment, and they also reported that spermatozoa have receptors for VEGF. While the function of VEGF in the male genital tract is unclear, it appears to influence the testicular microvasculature and the composition of seminal plasma. Taken together it might be that the heterozygous carriers of the *HRG* C633T SNP are favoured due to a more balanced regulation of angiogenesis during spermatogenesis through different pro- and/or anti-angiogenic actions depending on the *HRG* C633T SNP, and that this

regulation is suboptimal in the homozygous *HRG* 633T SNP men. It is also possible that HRG exerts a direct effect on the spermatozoa and not through its action as an adaptor molecule.

Our findings add to the growing literature on genetic reasons for infertility (Chen et al., 2015; El Inati et al., 2012; Ferlin and Foresta, 2014; Ge et al., 2015; Plaseski et al., 2012; Sato et al., 2015). It is well known that both autosomal and sex chromosome genes are involved in the complex regulation of spermatogenesis (Guo et al., 2012; Singh and Jaiswal, 2011). However, non-syndromic autosomal gene defects may also result in male infertility, with mutations in the *SPATA16*, *PICK1* and *DPY192L2* genes and SNPs in the *PRMT6*, *PEX10* and *SOX5* genes being associated with sperm defects or spermatogenesis (El Inati et al., 2012). Male autosomal chromosome variation, i.e. differences in size or staining of chromosome segments, has been negatively associated with fertilization rate (Liang et al., 2014) and clinical pregnancy rate (Guo et al., 2012; Singh and Jaiswal, 2011) following IVF, whereas such findings in women had no influence. In addition, a polymorphism in the paternal or maternal *methylenetetrahydrofolate reductase* (*MTHFR*) gene was associated with recurrent pregnancy loss (Vanilla et al., 2015), Ozdemir et al., 2012).

A limitation with this study is the low number of couples with male factor infertility as the single reason for treatment, which makes it impossible to draw any final conclusions, but the results are interesting and generate well-defined hypotheses to be investigated in a prospective cohort study with a larger setting in the future. In addition to the many reasons for infertility, the complex nature of these types of studies is underscored by the fact that each individual of the couple contributes with intricate genetic, proteomic and metabolic differences that interact (Kovac et al., 2013), and as noted in the present study, sub-fertility cannot be simply explained by one single causative polymorphism. Apparently, the exact role of HRG in reproduction remains to be investigated, but we can add to the puzzle that infertile couples where the male partner is homozygous for the *HRG* 633T SNP have lower semen quality and are at risk of not having a successful IVF treatment.

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Appendix: Supplementary material

Supplementary data to this article can be found online at [doi:10.1016/j.rbmo.2016.05.004](https://doi.org/10.1016/j.rbmo.2016.05.004).

References

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011. The Istanbul consensus workshop

- on embryo assessment: proceedings of an expert meeting. *Hum. Reprod.* 26, 1270–1283.
- Andersen, J.M., Herning, H., Aschim, E.L., Hjelmessaeth, J., Mala, T., Hanevik, H.I., Bungum, M., Haugen, T.B., Witczak, O., 2015. Body mass index is associated with impaired semen characteristics and reduced levels of anti-mullerian hormone across a wide weight range. *PLoS ONE* 10, e0130210.
- Bazer, F.W., Wu, G., Spencer, T.E., Johnson, G.A., Burghardt, R.C., Bayless, K., 2010. Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol. Hum. Reprod.* 16, 135–352.
- Brown, L.F., Yeo, K.T., Berse, B., Morgentaler, A., Dvorak, H.F., Rosen, S., 1995. Vascular permeability factor (vascular endothelial growth factor) is strongly expressed in the normal male genital tract and is present in substantial quantities in semen. *J. Urol.* 154, 576–579.
- Chen, X.Y., Chen, P., Xu, C., Zhang, X.H., 2015. Association of DAZL A260G and A386G polymorphisms with oligozoospermia- or azoospermia-induced male infertility: a meta-analysis]. *Zhonghua Nan Ke Xue.* 21, 345–356.
- Cotton, L.M., O'bryan, M.K., Hinton, B.T., 2008. Cellular signaling by fibroblast growth factors (FGFs) and their receptors (FGFRs) in male reproduction. *Endocr. Rev.* 29, 193–216.
- Cummins, J.M., Jequier, A.M., Kan, R., 1994. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? *Mol. Reprod. Dev.* 37, 345–362.
- Dixelius, J., Olsson, A.K., Thulin, A., Lee, C., Johansson, I., Claesson-Welsh, L., 2006. Minimal active domain and mechanism of action of the angiogenesis inhibitor histidine-rich glycoprotein. *Cancer Res.* 66, 2089–2097.
- Ebisch, I.M., Thomas, C.M., Wetzels, A.M., Willemsen, W.N., Sweep, F.C., Steegers-Theunissen, R.P., 2008. Review of the role of the plasminogen activator system and vascular endothelial growth factor in subfertility. *Fertil. Steril.* 90, 2340–2350.
- El Inati, E., Muller, J., Viville, S., 2012. Autosomal mutations and human spermatogenic failure. *Biochim. Biophys. Acta* 1822, 1873–1879.
- Elenis, E., Lindgren, K.E., Karypidis, H., Skalkidou, A., Hosseini, F., Bremme, K., Landgren, B.M., Skjoldebrand-Sparre, L., Stavreus-Evers, A., Sundstrom-Poromaa, I., Akerud, H., 2014. The histidine-rich glycoprotein A1042G polymorphism and recurrent miscarriage: a pilot study. *Reprod. Biol. Endocrinol.* 12, 70.
- Ergun, S., Kilic, N., Fiedler, W., Mukhopadhyay, A.K., 1997. Vascular endothelial growth factor and its receptors in normal human testicular tissue. *Mol. Cell. Endocrinol.* 131, 9–20.
- Esteves, S.C., Hamada, A., Kondray, V., Pitchika, A., Agarwal, A., 2012. What every gynecologist should know about male infertility: an update. *Arch. Gynecol. Obstet.* 286, 217–229.
- Febbraio, M., Hajjar, D.P., Silverstein, R.L., 2001. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J. Clin. Invest.* 108, 785–791.
- Ferlin, A., Foresta, C., 2014. New genetic markers for male infertility. *Curr. Opin. Obstet. Gynecol.* 26, 193–198.
- Ge, S.Q., Griffin, J., Liu, L.H., Aston, K.I., Simon, L., Jenkins, T.G., Emery, B.R., Carrell, D.T., 2015. Associations of single nucleotide polymorphisms in the Pygo2 coding sequence with idiopathic oligospermia and azoospermia. *Genet. Mol. Res.* 14, 9053–9061.
- Gorgani, N.N., Parish, C.R., Easterbrook Smith, S.B., Altin, J.G., 1997. Histidine-rich glycoprotein binds to human IgG and C1q and inhibits the formation of insoluble immune complexes. *Biochemistry* 36, 6653–6662.
- Guo, T., Qin, Y., Gao, X., Chen, H., Li, G., Ma, J., Chen, Z.J., 2012. The role of male chromosomal polymorphism played in spermatogenesis and the outcome of IVF/ICSI-ET treatment. *Int. J. Androl.* 35, 802–809.
- Hansson, B., Westerberg, L., 2002. On the correlation between heterozygosity and fitness in natural populations. *Mol. Ecol.* 11, 2467–2474.
- Hennis, B.C., Van Boheemen, P.A., Wakabayashi, S., Koide, T., Hoffmann, J.J., Kievit, P., Dooijewaard, G., Jansen, J.G., Kluft, C., 1995. Identification and genetic analysis of a common molecular variant of histidine-rich glycoprotein with a difference of 2 kD in apparent molecular weight. *Thromb. Haemost.* 74, 1491–1496.
- Jodar, M., Sendler, E., Krawetz, S.A., 2016. The protein and transcript profiles of human semen. *Cell Tissue Res.* 363, 85–96.
- Jones, A.L., Hulett, M.D., Altin, J.G., Hogg, P., Parish, C.R., 2004. Plasminogen is tethered with high affinity to the cell surface by the plasma protein, histidine-rich glycoprotein. *J. Biol. Chem.* 279, 38267–38276.
- Jones, A.L., Hulett, M.D., Parish, C.R., 2005. Histidine-rich glycoprotein: a novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunol. Cell Biol.* 83, 106–118.
- Kamath, M.S., Bhattacharya, S., 2012. Demographics of infertility and management of unexplained infertility. *Best Pract. Res. Clin. Obstet. Gynaecol.* 26, 729–738.
- Kassar, O., McMahon, S.A., Thompson, R., Botting, C.H., Naismith, J.H., Stewart, A.J., 2014. Crystal structure of histidine-rich glycoprotein N2 domain reveals redox activity at an interdomain disulfide bridge: implications for angiogenic regulation. *Blood* 123, 1948–1955.
- Kovac, J.R., Pastuszak, A.W., Lamb, D.J., 2013. The use of genomics, proteomics, and metabolomics in identifying biomarkers of male infertility. *Fertil. Steril.* 99, 998–1007.
- Laanpere, M., Altmae, S., Kaart, T., Stavreus-Evers, A., Nilsson, T.K., Salumets, A., 2011. Folate-metabolizing gene variants and pregnancy outcome of IVF. *Reprod. Biomed. Online* 22, 603–614.
- Lash, G.E., Innes, B.A., Drury, J.A., Robson, S.C., Quenby, S., Bulmer, J.N., 2012. Localization of angiogenic growth factors and their receptors in the human endometrium throughout the menstrual cycle and in recurrent miscarriage. *Hum. Reprod.* 27, 183–195.
- Lee, C., Dixelius, J., Thulin, A., Kawamura, H., Claesson-Welsh, L., Olsson, A.K., 2006. Signal transduction in endothelial cells by the angiogenesis inhibitor histidine-rich glycoprotein targets focal adhesions. *Exp. Cell Res.* 312, 2547–2556.
- Lerch, P.G., Nydegger, U.E., Kuyas, C., Haerberli, A., 1988. Histidine-rich glycoprotein binding to activated human platelets. *Br. J. Haematol.* 70, 219–224.
- Liang, J., Zhang, Y., Yu, Y., Sun, W., Jing, J., Liu, R., 2014. Effect of chromosomal polymorphisms of different genders on fertilization rate of fresh IVF-ICSI embryo transfer cycles. *Reprod. Biomed. Online* 29, 436–444.
- Lindgren, K.E., Karehed, K., Karypidis, H., Hosseini, F., Bremme, K., Landgren, B.M., Skjoldebrand-Sparre, L., Stavreus-Evers, A., Sundstrom-Poromaa, I., Akerud, H., 2013. Histidine-rich glycoprotein gene polymorphism in patients with recurrent miscarriage. *Acta Obstet. Gynecol. Scand.* 92, 974–977.
- Nasah, B.T., Cox, J.N., 1978. Vascular lesions in testes associated with male infertility in Cameroon. Possible relationship to parasitic disease. *Virchows Arch. A. Pathol. Anat. Histol.* 377, 225–236.
- Nordqvist, S., Karehed, K., Hambiliki, F., Wanggren, K., Stavreus-Evers, A., Akerud, H., 2010. The presence of histidine-rich glycoprotein in the female reproductive tract and in embryos. *Reprod. Sci.* 17, 941–947.
- Nordqvist, S., Karehed, K., Stavreus-Evers, A., Akerud, H., 2011a. Histidine-rich glycoprotein polymorphism and pregnancy outcome: a pilot study. *Reprod. Biomed. Online* 23, 213–219.
- Nordqvist, S., Karehed, K., Stavreus-Evers, A., Akerud, H., 2011b. Histidine-rich glycoprotein polymorphism and pregnancy outcome: a pilot study. *Reprod. Biomed. Online* 23, 213–219.
- Nordqvist, S., Karehed, K., Skoog Svanberg, A., Menezes, J., Akerud, H., 2015. Ovarian response is affected by a specific histidine-rich glycoprotein polymorphism: a preliminary study. *Reprod. Biomed. Online* 30, 74–81.

- Obermair, A., Obruca, A., Pohl, M., Kaider, A., Vales, A., Leodolter, S., Wojta, J., Feichtinger, W., 1999. Vascular endothelial growth factor and its receptors in male fertility. *Fertil. Steril.* 72, 269–275.
- Olsson, A.K., Larsson, H., Dixelius, J., Johansson, I., Lee, C., Oellig, C., Bjork, I., Claesson-Welsh, L., 2004. A fragment of histidine-rich glycoprotein is a potent inhibitor of tumor vascularization. *Cancer Res.* 64, 599–605.
- Ozdemir, O., Yenicesu, G.I., Silan, F., Koksak, B., Atik, S., Ozen, F., Gol, M., Cetin, A., 2012. Recurrent pregnancy loss and its relation to combined parental thrombophilic gene mutations. *Genet. Test Mol. Biomarkers.* 16, 279–286.
- Pinborg, A., Gaarslev, C., Hougaard, C.O., Nyboe Andersen, A., Andersen, P.K., Boivin, J., Schmidt, L., 2011. Influence of female bodyweight on IVF outcome: a longitudinal multicentre cohort study of 487 infertile couples. *Reprod. Biomed. Online* 23, 490–499.
- Plaseski, T., Noveski, P., Popeska, Z., Efremov, G.D., Plaseska-Karanfilska, D., 2012. Association study of single-nucleotide polymorphisms in FASLG, JMJD1A, LOC203413, TEX15, BRDT, OR2W3, INSR, and TAS2R38 genes with male infertility. *J. Androl.* 33, 675–683.
- Poon, I.K., Hulett, M.D., Parish, C.R., 2010. Histidine-rich glycoprotein is a novel plasma pattern recognition molecule that recruits IgG to facilitate necrotic cell clearance via FcγRI on phagocytes. *Blood* 115, 2473–2482.
- Sato, Y., Tajima, A., Tsunematsu, K., Nozawa, S., Yoshiike, M., Koh, E., Kanaya, J., Namiki, M., Matsumiya, K., Tsujimura, A., Komatsu, K., Itoh, N., Eguchi, J., Imoto, I., Yamauchi, A., Iwamoto, T., 2015. An association study of four candidate loci for human male fertility traits with male infertility. *Hum. Reprod.* 30, 1510–1514.
- Simantov, R., Febbraio, M., Crombie, R., Asch, A.S., Nachman, R.L., Silverstein, R.L., 2001. Histidine-rich glycoprotein inhibits the antiangiogenic effect of thrombospondin-1. *J. Clin. Invest.* 107, 45–52.
- Simantov, R., Febbraio, M., Silverstein, R.L., 2005. The antiangiogenic effect of thrombospondin-2 is mediated by CD36 and modulated by histidine-rich glycoprotein. *Matrix Biol.* 24, 27–34.
- Singh, K., Jaiswal, D., 2011. Human male infertility: a complex multifactorial phenotype. *Reprod. Sci.* 18, 418–425.
- Thornton, P., Douglas, J., 2010. Coagulation in pregnancy. *Best Pract. Res. Clin. Obstet. Gynaecol.* 24, 339–352.
- Tocci, A., Lucchini, C., 2010. WHO reference values for human semen. *Hum. Reprod. Update* 16, 559. Author reply 559.
- Vanilla, S., Dayanand, C.D., Kotur, P.F., Kutty, M.A., Vegi, P.K., 2015. Evidence of paternal N5, N10-Methylenetetrahydrofolate Reductase (MTHFR) C677T gene polymorphism in couples with recurrent spontaneous abortions (RSAs) in Kolar District- A South West of India. *J. Clin. Diagn. Res.* 9, BC15–BC18.
- Venkatesh, T., Suresh, P.S., Tsutsumi, R., 2014. New insights into the genetic basis of infertility. *Appl. Clin. Genet.* 7, 235–243.
- Vu, T.T., Stafford, A.R., Leslie, B.A., Kim, P.Y., Fredenburgh, J.C., Weitz, J.I., 2011. Histidine-rich glycoprotein binds fibrin(ogen) with high affinity and competes with thrombin for binding to the gamma'-chain. *J. Biol. Chem.* 286, 30314–30323.
- Wakabayashi, S., 2013. New insights into the functions of histidine-rich glycoprotein. *Int. Rev. Cell. Mol. Biol.* 304, 467–493.
- Wakabayashi, S., Koide, T., 2011. Histidine-rich glycoprotein: a possible modulator of coagulation and fibrinolysis. *Semin. Thromb. Hemost.* 37, 389–394.
- Wake, H., Mori, S., Liu, K., Takahashi, H.K., Nishibori, M., 2009. Histidine-rich glycoprotein inhibited high mobility group box 1 in complex with heparin-induced angiogenesis in matrigel plug assay. *Eur. J. Pharmacol.* 623, 89–95.
- Waylen, A.L., Metwally, M., Jones, G.L., Wilkinson, A.J., Ledger, W.L., 2009. Effects of cigarette smoking upon clinical outcomes of assisted reproduction: a meta-analysis. *Hum. Reprod. Update* 15, 31–44.
- Wilhelm, M., Schlegl, J., Hahne, H., Moghaddas Gholami, A., Lieberenz, M., Savitski, M.M., Ziegler, E., Butzmann, L., Gessulat, S., Marx, H., Mathieson, T., Lemeer, S., Schnatbaum, K., Reimer, U., Wenschuh, H., Mollenhauer, M., Slotta-Huspenina, J., Boese, J.H., Bantscheff, M., Gerstmair, A., Faerber, F., Kuster, B., 2014. Mass-spectrometry-based draft of the human proteome. *Nature* 509, 582–587.

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