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The relevance of ANXA5 genetic variants on male fertility

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Abstract

Purpose To investigate the effect of the anticoagulation factor annexin A5 on male fertility and to provide perspective on the influence of members of the coagulation cascade on fertility.

Methods Patients with normozoospermia and with unexplained severe oligozoospermia were retrospectively selected and their genomic DNA sequenced for the promoter region of *ANXA5*. The genotypes proportions and the odds ratio for carriership of the haplotype M2 were compared between the groups and population control. The clinical data used were gathered from parameters determined during routine clinical assessment and were compared between carriers and non-carriers within the patient groups.

Results The carrier rates for the haplotype M2/*ANXA5* were of 25.73%, 20.81%, and 15.3% in the severe oligozoospermic, the normozoospermic, and the general population control groups, respectively. The OR between patients groups was of 1.31 (95% CI 0.88 to 1.96 $p = 0.176$). Oligozoospermic and normozoospermic patients compared with the control group had an OR of 1.9 (95% CI 1.33 to 2.73 $p < 0.001$) and 1.45 (95% CI 0.99 to 2.10 $p = 0.054$) respectively. The clinical parameters that differed between the carriers and non-carriers of the haplotype M2/*ANXA5* were prolactin, α-glucosidase, and fructose. The differences were only statistically significant in the normozoospermic group.

Conclusions Athough the infertile patient groups had a higher prevalence of promoter variants, we could not demonstrate any biologically relevant effect of lower levels of annexin A5 on most male fertility parameters. A deficiency in an anticoagulation factor does not seem to impact male fertility.

Keywords Annexin A5 · Male fertility · Haplotype M2 · *ANXA5* · Sperm count · Coagulation cascade

Introduction

Inherited mutations in the coagulation cascade increase the risk of pregnancy loss due to placental vascular thrombosis [1]. Cohn and colleagues considered the possibility of a coagulation factor alteration leading to an evolutionary increase in male fertility in 2010 as an explanation for the observed high frequency of factor V Leiden (FVL), the most common genetic cause of thrombosis, in the general population [2]. This hypothesis was based on the observation of increased sperm count and total number of motile sperm in men heterozygous for FVL [2]. However, Yapijakis and colleagues, while looking at the frequency of FVL in men with normal and low sperm counts, could not confirm this finding nor could they identify any association between factor II G20210A, another primary coagulation factor mutation, and male fertility [3].

Annexin A5 is an anticoagulation protein consisting of 320 amino acids with a molecular weight of 36 kDa. Annexin A5 is coded by the *ANXA5* gene located on the human chromosome 4q27. The proximal core of the promoter region has two common variations, known as haplotypes M1 and M2. The haplotype M1/*ANXA5* comprises two consecutive single-nucleotide polymorphisms (SNPs): 1A ➔ C (A-448C, rs28717001) and 27T ➔ C (T-422C, rs28651243). The haplotype M2/*ANXA5* is a combination of the SNPs present on the haplotype M1/*ANXA5* with the addition of two new SNPs: -19G ➔ A (G-467A, rs112782763_) and 76 G ➔ A (G-373A, rs113588187) [4]. These variants decrease the *ANXA5* gene promoter region activity [4] and mRNA expression [5, 6], with the influence of the haplotype M1/*ANXA5* being less pronounced than the one from haplotype M2/*ANXA*.

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Furthermore, the carrier status of the haplotype M2/*ANXA5* is a significant risk factor for recurrent pregnancy loss during the early stages of pregnancy [7]. Interestingly, the risk factor status of the haplotype M2/*ANXA5* is independent of the parental origin; i.e., there is an equal contribution of the maternal and the paternal allele in the pregnancy loss risk indicating that it harms on the placental level [7–9].

Annexin A5 has been detected in proteomic studies of several organs of the human male reproductive system, including the testes [10], epididymis [11], prostate [12], and sperm [13]. Furthermore, annexin A5 expression has been detected in the epididymis, testis, and endocrine organs of rats [14, 15]. However, the influence of annexin A5 on semen parameters has only been studied in rabbits, where in seminal plasma annexin A5 is the main protein component. In rabbits, annexin A5 influences sperm concentration, motility, and morphology [16]. In men, the role of annexin A5 in men is still enigmatic.

Therefore, this study investigates the relationship of the genetic variants of *ANXA5* gene promoter region with male fertility parameters and aims to provide a new perspective on whether defects in the coagulation cascade influence male fertility.

Methods

Study cohorts

This study was approved by the Ethics Commission of the Westphalia-Lippe Medical Association and the Medical Faculty of the University of Muenster (2012-246-f-S). All patients provided signed informed consent to have their clinical data evaluated and their donated DNA genetically analyzed. The study group was retrospectively selected from patients that consulted the Center of Reproductive Medicine and Andrology. Selected patients had unexplained severe oligozoospermia ($n = 272$), which is defined as sperm concentration < 5 million spermatozoa per ml, or normozoospermia ($n = 269$), which is defined as ≥ 39 million spermatozoa in the ejaculate, of which ≥ 32% are progressive motile and ≥ 4% have normal morphology according to the current World Health Organization reference ranges [17]. Patients were excluded if a condition could explain their infertility, i.e., chemo- or radiotherapy because of oncological disease, varicocele, and cryptorchidism. A general population control sample of healthy subjects was drafted from the PopGen biobank from 2007, identified through official population registers [18]. The sample that has been described previously [8] comprised an approximately equal number of men and women with unknown fertility status that distributed among three age groups: 18–30; 30–50; 50–80 years.

Genotyping of ANXA5 proximal promoter region

Patient groups were genotyped by using genomic DNA extracted from peripheral blood and employing Sanger sequencing of the relevant amplicon in line with established protocols [4, 19]. The accuracy of the genotyping was verified by a random blinded inclusion of 2% repeated measures. Genotypes were scored in table format and four-digit coded for further processing.

Hormonal and semen analyses

The data from the patient groups were gathered retrospectively from the parameters assessed as part of the routine clinical procedure. The available hormonal parameters were for FSH, LH, testosterone, prolactin, and estradiol. The sperm parameters evaluated were semen volume, sperm concentration, total count, progressive motility, vitality, and concentrations of accessory gland markers (α-glucosidase for the epididymis, fructose for the seminal glands, and zinc for the prostate).

Serum concentrations of FSH, LH, estradiol, and prolactin were determined by immunofluorometric assays and serum testosterone by a commercial ELISA kit.

Semen samples were obtained by masturbation after 0–10 days of abstinence and all the analyses were performed according to the WHO Laboratory Manual, 2010 [17]. Sperm concentration was analyzed with Neubauer-improved chambers, and the total sperm count was calculated by multiplying semen volume by sperm concentration. Motility was assessed on a heated microscope stage. Neutral α-glucosidase, fructose, and zinc were measured by multi-well spectrophotometric assays.

Statistical analysis

Statistical analyses were carried out using the SPSS package (SPSS version 23.0, Chicago, USA). The differences in the genotype distributions were evaluated using Z test with a subsequent Bonferroni adjustment for p values. Odds ratios (OR) and 95% confidence intervals (CI) were calculated and differences in the number of carriers were evaluated using Pearson's chi-square test. Possible deviations from the Hardy-Weinberg equilibrium (HWE) were calculated using a Monte-Carlo Markov chain (MCMC) implementation of an exact test, part of the Genepop package [20]. Comparisons of the reproductive parameters between the carriers and non-carriers of the haplotype M2 were carried out using the non-parametric Mann-Whitney U test since the parameters were not normally distributed. Due to the exploratory nature of the comparisons of the reproductive parameters, a correction for multiple testing was not applied. Adjusted and non-adjusted p values smaller or equal 0.05 were considered significant.

Results

The distribution of genotypes among the patient groups and control group is summarized in Table 1. The proportion of patients having the non-mutated promoter region (N/N), presenting the haplotype M1 and the haplotype M2 (M1/M2), and heterozygous carriers of the haplotype M2 (N/M2, M1/M2) were significantly different when compared with the general population control. The total M2 carrier rates were 25.73% for oligozoospermic and 20.81% for the normozoospermic patients (Table 1). The possible association of M2 carrier status with sperm count was then assessed in the two comparably sized groups of severe oligozoospermic and normozoospermic patients. Both groups were in HWE at $p = 0.879$ and $p = 07147$, respectively. The comparison of both patient groups yielded an OR of 1.31 (95% CI 0.88 to 1.96), not achieving significance ($p = 0.176$). The comparison between the normozoospermic group with the general population control group revealed an OR of 1.45 (95% CI 0.99 to 2.10), also not significant ($p = 0.054$). However, when comparing the oligozoospermic patients with the general population control group, the OR was 1.9 (95% CI 1.33 to 2.73), achieving statistical significance ($p < 0.001$).

The hormonal and semen parameters are summarized in Table 2. In the normozoospermic patient group, the values of all parameters were within the normal range independently of the M2/*ANXA5* haplotype carriership status. The only statistically significant differences between the carriers and non-carriers of the haplotype M2 could be observed in the prolactin, fructose, and α-glucosidase levels. The median prolactin level in the non-carrier group was 177.5 mU/l and in the haplotype M2 carrier group was 162.5 mU/l. The median fructose level in the non-carrier group was 46 μmol and in the haplotype M2 carrier group was 57.8 μMol. The median α-glucosidase levels were 80.8 mU and 105.4 mU in the non-carrier and haplotype M2 carrier group, respectively. In the oligozoospermic patients, there was no statistical difference between the carriers and non-carriers of the haplotype M2 in any parameter. In the oligozoospermic group, there was an apparent decrease in the median prolactin level and an increase in median fructose and α-glucosidase levels. However, these variations are minimal and not statistically significant.

Discussion and conclusions

Our study aimed to investigate, for the first time, the influence of the annexin A5 on male fertility. To do so, we investigated the enrichment of the *ANXA5* genetic variants in patients of a reproductive care facility. The proportions of the haplotypes in our patients were significantly different when compared with the general populations control, but not different between severe oligozoospermic and normozoospermic patients. The patient groups presented a lower proportion of non-mutated promoter region of the *ANXA5*. This decrease was explained by a higher proportion of heterozygous carriers of the haplotype M2 and a higher proportion of carriers of the haplotype M1/*ANXA5* and the haplotype M2/*ANXA5* concurrently. Our evaluation of the carriership of the haplotype *M2/ANXA5* showed a non-significant increase in carriers of the M2/*ANXA5* haplotype in oligozoospermic patients when compared with normozoospermic patients and a significant increase when compared with the general population control. The high prevalence of the haplotype M2 on our patients led us to analyze the influence of the haplotype M2/*ANXA5* on the clinical parameters and the possibility of a phenotype existence. Despite the enrichment in the oligozoospermic population, the haplotype M2/*ANXA5* did not influence sperm parameters. Regarding the remaining clinical parameters, we could find statistically significant differences between the carriers and non-carriers of the haplotype M2/*ANXA5* in normozoospermic patients for prolactin, fructose and α-glucosidase.

Table 1 Genotype frequencies of ANXA5 promoter haplotypes in severe oligozoospermic patients, normozoospermic patients, and control group

	Severe Oligozoospermia ($n = 272$)	Normozoospermia ($n = 269$)	PopGen controls ($n = 533$)
N/N	64.0 (174)[a]	68.8 (185)[a]	77.9 (415)[b]
N/M1	10.3 (28)[a]	10.0 (27)[a]	6.6 (35)[a]
M1/M1	0 (0)[a]	0.4 (1)[a]	0.2 (1)[a]
M1/M2	1.5 (4)[a]	1.8 (5)[a]	0.9 (5)[b]
N/M2	23.2 (63)[a]	18.2 (49)[a,b]	13.5 (72)[b]
M2/M2	1.1 (3)[a]	0.7 (2)[a]	0.9 (5)[a]
M2 carrier rate	25.7 (70)[a]	20.8 (56)[a,b]	15.3 (82)[b]

The data is presented as % (*n*). For each row, cells with different superscript letters have significantly different column proportions ($p < 0.05$)

N the wild-type promoter sequence, *M1* the haplotype M1 promoter sequence, *M2* the haplotype M2 promoter sequence

Table 2 Clinical parameters of carriers and non-carriers of the haplotype M2/ANXA5 from the oligozoospermic and normozoospermic patients

	Severe oligozoospermia ($n = 272$)			Normozoospermia ($n = 269$)		
	Non-carriers ($n = 202$)	M2 carriers ($n = 70$)	p value	Non-carriers ($n = 213$)	M2 carriers ($n = 56$)	p value
FSH (U/l)	6.4 (3.9–10.8)	5.85 (3.7–10.35)	0.584	3.1 (2.3–4.3)	3.45 (2.6–4.5)	0.145
LH (U/l)	3.7 (2.8–5.65)	4.4 (3.05–5.45)	0.499	2.8 (2.05–3.7)	3.15 (2.2–3.875)	0.186
Testosterone (nmol/l)	14.4 (10.65–18.7)	15.35 (11.8–18.625)	0.405	15.6 (12.1–18.4)	15.95 (15.825–19.1)	0.383
Prolactin (mU/l)	180 (133–221.5)	156 (118–222.5)	0.131	177.5 (131–236.25)	162.5 (120.25–194)	0.047*
Estradiol (pmol/l)	75 (61–89)	80 (64–101)	0.176	69 (56–79.75)	67 (58–86)	0.622
Volume (ml)	3.2 (2.4–4.33)	3.4 (2.6–4.2)	0.712	3.4 (2.6–4.65)	3.75 (3–5)	0.092
Concentration (Mill./ml)	1 (0.4–1.9)	1 (0.4–1.85)	0.887	59 (37.2–101.3)	51.3 (34.625–84)	0.270
Total count (Mill.)	3.25 (1.3–6.13)	3.5 (1.3–6)	0.811	225.8 (141.25–353.3)	205.6 (134.85–307.175)	0.541
Progressive motility (%)	29 (17.75–41)	30 (18–38.25)	0.676	53 (49–57)	53 (49.25–57)	0.801
Live cells (%)	60 (50–68)	55 (42–64)	0.062	69 (63–73)	70 (65–75)	0.133
α-Glucosidase (mU)	48 (27.25–69.55)	47 (29.7–81.05)	0.471	80.8 (55.4–118.3)	105.4 (59.9–155)	0.016*
Fructose (μmol)	50.5 (28.5–78.1)	57.6 (35.4–81.3)	0.204	46 (30–71)	57.8 (43–79)	0.011*
Zinc (μmol)	5.4 (3.2–8.8)	5.55 (2.725–7.575)	0.611	6.2 (3.825–9.2)	7.2 (4.6–11.1)	0.131

The data is presented as mean (25–75th percentiles)

*p value is considered significant at $p < 0.05$

However, these differences were all still inside the normal range.

Our results in humans do not mirror those in rabbits that demonstrate the importance of annexin A5 on sperm parameters [16]. In rabbits, annexin A5 is the major seminal plasma protein [16]. Annexin A5 is thought to act as a decapacitating factor that holds rabbit sperm in a quiescent state during their storage in the female reproductive tract prior to ovulation, which only occurs after mating [16]. Annexin A5 has been listed in several proteomic studies, and it is present in the seminal human plasma, however not as the primary protein, which is albumin [13]. In humans, there is no defined sperm storage time, and annexin A5 does not seem to be crucial for human sperm parameters.

In our study, α-glucosidase was significantly increased in the carriers of the haplotype M2/*ANXA5*. α-Glucosidase is produced mainly by the epididymis and is a reliable marker of epididymal secretory function since it indicates tubule patency [21]. A low threshold for this marker was defined, and any value below indicates possible tubular occlusion [17]. However, no high threshold value has been set, and the significance of elevated secretion of α-glucosidase is unclear [21]. There is still a lack of knowledge on the biological role of both annexin A5 and α-glucosidase in the epididymis. No associations, until now, have been reported between fructose and annexin A5. Fructose reflects the secretory functions of the seminal vesicles and also only has a minimum threshold defined [17] . Therefore, any explanation for these associations observed would be highly speculative.

In humans, the relation between annexin A5 and prolactin does not seem to be the same as the one found in rats. Since 1992, Kawaminami and colleagues have studied the relationship between annexin A5 and prolactin in rats both in vivo and in vitro using rat pituitary primary cell cultures. However, their findings are inconsistent [22–25]. Nevertheless, annexin A5 generally decreases the secretion of prolactin in the studies mentioned above. However, our study showed that the presence of the haplotype M2/*ANXA5* (i.e., lower levels of annexin A5) was associated with a decrease in prolactin levels. Recent studies show that prolactin may play a role in male reproduction. However, further studies are needed for verification of those findings [26].

Our results revealed a higher proportion and prevalence of the haplotype M2/*ANXA5* in patients seeking reproductive care. Although there are evidences of association between some reproductive parameters with annexin A5, our study does not show any critical effect of lower levels of annexin A5 on these male fertility parameters. This finding parallels the study by Yapijakis and colleagues that reports no association between male infertility and genetic variants of two other coagulation factors: factor V Leiden and factor II G20210A [3]. Combined [3], our results contradict the proposed theory that an evolutionary advantage can be ascribed to male carriers of thrombophilia-related gene variants predisposing to pregnancy complications [2].

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