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Title: Association of polymorphisms in genes coding for antioxidant enzymes and human male infertility.

Running Title: Antioxidant capacity and male infertility

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Abstract

Purpose: While oxidative stress is thought to be an important cause of male infertility, primarily due to DNA and cell membrane damage, little is known about the genetic causes underlying suboptimal function of the seminal enzymatic antioxidant system. The aim of this study was to investigate the relationship of 4 potentially functional polymorphisms associated to oxidative stress pathway genes (superoxide dismutase - SOD2.lle58Thr and SOD2.rs4880, Catalase – CAT C-262T, glutathione peroxidase 1 - GPX1 Pro200Leu) and 2 null variants of the gluthathione S transferase (GSTT and GSTM) genes and infertility risk.

Methods: A case control study was conducted on 313 infertile patients and 80 fertile donors. Each ejaculate was subjected to a seminal analysis which included the classical parameters seminal volume, sperm concentration, sperm motility and sperm morphology, as well as sperm DNA fragmentation (patients only). Polymerase chain reaction-restriction fragment length polymorphism (PCR_RFLP) and PCR multiplex methods were carried out for genotyping.

Results: Statistically significant differences were found between fertile donors and infertile patients for SNP CAT C-262T; the CC genotype was related with a 2 fold increased risk of infertility (OR = 2.262; 95%CI = 1.369 - 3.733; P = 0.001) while the CT genotype was associated with a protective effect (OR = 0.401; 95%CI = 0.241 - 0.667; P = 0.001). Surprisingly, the SOD2 Ile58Thr SNP was not represented in the sample population, so it's frequency in the current population frequenting fertility clinics in Madrid may be very low.

Conclusions: Our results suggest that the CAT SNP C-262T is potentially associated with an increased risk of male infertility.

Introduction

Reactive oxygen species (ROS) are free radicals having at least one unpaired electron and include molecules such the hydroxyl ion [OH], superoxide ion [O_2], peroxyl radical [RO_2] or hydrogen peroxide [H_2O_2]. ROS originate from cellular reactions as by-products of the metabolism of oxygen and are normally neutralized by the homeostatic antioxidant systems. The ROS present in seminal plasma can originate from both endogenous or exogenous sources; endogenous sources may be derived from a high incidence of leukocytes in the seminal plasma and an overabundance of immature spermatozoa (sperm cells with residual cytoplasm) in the ejaculate of patients with varicocele [1-3]. ROS in seminal plasma may also originate from exogenous sources such as exposure to ionising radiation, cytotoxins or poor lifestyle choice, including cigarette smoking or excessive alcohol consumption [4].

While the presence of ROS in seminal plasma is normally balanced by homeostatic antioxidant systems that facilitate an appropriate level of ROS required for normal physiological processes such as sperm capacitation, hyperactivation, acrosome reaction and sperm-oocyte fusion, in some circumstances, unbalanced REDOX potential (a measure of oxidative stress defined as all known and unknown contributors of oxidative stress and not limited to a specific constituent as is the case in ROS or TAC assays), may arise due to an elevated production of ROS that is beyond the capacity of the animal's inherent antioxidant systems to cope with. Under such circumstances, high levels of ROS in the seminal plasma have the potential to not only cause sperm DNA damage, but also lipid peroxidation, sperm motility and membrane fluidity reduction and apoptosis [5-6].

The enzymatic antioxidant systems in seminal plasma are a group of enzymes that include, manganese superoxide dismutase (SOD2), catalase (CAT), glutathione peroxidase 1

(GXP1) and gluthathione S transferase (GST) [7]. SOD2 is an enzyme that catalyses the detoxification of superoxide radicals in the mitochondrion [8-10]. Catalase has the capacity to detoxify H_2O_2 by converting it into H_2O and O_2 [11,12]. The GXP1 enzyme is related to the final electron transporter and neutralizes peroxide radicals into H_2O [13], whereas GST conjugates toxic electrophiles and other intermediates, with glutathione negating their toxicity [14].

Although these enzymes appear to be conserved phylogenetically, species and individual specific polymorphisms may still produce changes in regard to their respective activities and, therefore, may be useful in understanding the underlying origins of idiopathic infertility. Hence, the aim of the current study was to elucidate and establish a genetic basis to investigate the relationship of enzymes associated with antioxidant system failure, conventional parameters of the WHO accredited sperm analysis and male infertility. To examine this hypothesis, we compared the incidence of 6 potentially functional polymorphisms thought to be linked to oxidative stress pathway genes (SOD2, CAT, GXP1, GSTM and GSTT) in infertile patients and donor controls with proven fertility attending a Spanish infertility clinic.

Materials and methods

Subjects and sample collection

The study was approved by the Bioethics Committee of the Universidad Autónoma de Madrid (CEI 60-1058). Semen samples from a total of 393 subjects (patients and donors) attending a Spanish Fertility Clinic were analysed consisting of 313 infertile patients undergoing infertility IVF/ICSI treatment and 80 donors with proven fertility who had already fathered at least one child. The patient cohort consisted of individuals that showed no evidence of any other fertility related diseases, such us prostate cancer, cryptorchidism, varicocele, diabetes, seminal

infections or karyotype abnormalities. Each male donated 1 mL (patient cohort) or 0.5 mL (donor cohort) of semen that was obtained by masturbation after at least 4 days of abstinence; this abstinence period was based on the standard protocol used in the collaborating infertility clinic.

Seminal analysis

Each ejaculate was subjected to a classical seminal analysis approximately 30 min after liquefaction based on the recommendations and semen evaluation protocols and standards of the World Health Organization (2010) [15]; parameters analysed included seminal volume, sperm concentration, sperm motility and sperm morphology.

Sperm DNA fragmentation

Sperm DNA fragmentation (SDF) was assessed for all the samples in the patient cohort using the Sperm Chromatin Dispersion Assay (SCD; Halosperm Kit, Halotech DNA, Madrid, Spain) according to the manufacturer's instructions. Processed slides were stained with SYBR-Green (SYBR-Green 10000x, Cat No S7563, Thermo Fisher Scientific, Brawnschweig, Germany), mounted with Vectashield (Vectashield Mounting Medium, Cat No H-1000, Vector Laboratories, Burlingame,USA) and observed with epifluorescence microscopy (Leica DMRB, Leica-Mycrosystems, Wetzlar, Germany).

DNA extraction

DNA extraction was performed using the phenol-chloroform method with proteinase-K treatment. Briefly, 500 µL of liquefied semen was centrifuged at 1800 rpm and the pellet re-

suspended in 100 µL of seminal plasma. This sample was then incubated overnight in a solution consisting of 8 µL 10 mg mL^{-1} proteinase K (Proteinase K Recombinant PCR Grade, Cat No 03 115 887 001, Roche, Mannheim, Germany), 8µL of 1M DTT and 100µL extraction buffer (20mM Tris-Cl, 20mM EDTA, 200mM NaCl, 4% SDS). Thereafter, 216 µL of phenol (Phenol/Chloroform/Isoamyl alcohol 25:24:1, Cat No 327115000, Acros Organics, New Jersey, USA) was added to the semen sample and the mixture agitated on a mechanical shaker for 2 min after which it was centrifuged at 8000 rpm for 10 min. The upper aqueous phase was then recovered and the same operation performed with 200 µL of chloroform:isoamil 24:1 (Chloroform reagent grade - Cat No CL02031000, Scharlab, Spain; Isoamylalkohol >98% - Cat No 818969, Merck, Munich, Germany). The upper aqueous phase was then recovered once more to which 250 µL of cold (-20 ºC) 100% ethanol was also added. This solution was gently shaken until the DNA precipitated and the sample left to stand overnight at -20 ºC. The DNA was then washed with cold (-20 ºC) 70% ethanol and air dried. To eliminate contaminating RNA, the pellet was re-suspended in 50 µL of TRIS-EDTA buffer with 0.5 µL of 1mg mL^{-1} RNAse (RNase DNase free, Cat No 11 119 915 001, Roche, Mannheim, Germany) and incubated for 2 h at 37 ºC. Finally, the concentration of the extracted DNA was determined using a NanoDrop ND-1000 (Thermo Scientific, Brawnschweig, Germany) before diluting to a concentration of 50 ng μL^{-1} and stored at 4 ºC.

Genotype analysis

SOD2 Ile58Thr, SOD2 Val16Ala (rs4880), CAT C-262T (rs1001179) and GXP1 Pro200Leu (rs1050450) genotypes were determined using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. PCR products were then digested with the restriction enzymes EcoRV, HaeIII, SmaI and HaeIII, respectively (FastDigest Restriction Enzymes, Cat Nos FD0303, FD 0154, FD0664, Thermo Scientific, Brawnschweig,

Germany). The respective primer sequences and the restriction enzymes are shown in Table 1. GSTM1-GSTT1 genotyping was performed using a PCR multiplex protocol [16]. Briefly, 3 sets of primers where used in an unique PCR mix: forward 5'-GAACTCCCTGAAAAGCTAAAGC-3' and reverse 5'-GTTGGGCTCAAATATACGGTTT-3' for GSTM1, forward 5'-TTCCTTACTGGTCCTCACATCTC-3' and reverse 5'-TCACCGGATCATGGCCAGCA-3' for GSTT1 and forward 5'-GCCCTCTGCTAACAAGTCCTAC-3' and reverse 5'-GCCCTAAAAAGAAAATCGCCAATC-3' for albumin, as an internal control. PCR product sizes were 210 bp, 480 bp and 350 bp respectively.

Statistical analysis

Statistical analysis was performed using the SPSS 22 software for Windows (SPSS Inc., Chicago. USA). Associations between categorical variables (genotype frequencies and odds ratios) were assessed by a chi-square test. Continuous variables (seminal parameters) were compared between groups using non-parametric tests (Mann-Whitney U-test - comparison between 2 groups and Kruskal-Wallis - comparison between more than 2 groups). Results were expressed as odds ratio (OR) with 95% confidence intervals (CI). A *P* value of <0.05 was considered statistically significant.

Results

The demographic and clinical profiles of the patient and donor cohorts analysed in this study are shown in Table 2; as expected, significant differences between both cohorts were obtained for almost all seminal parameters with the donor cohort showing the best seminal quality.

Genotype frequencies of each of the polymorphisms in the patient and donor cohort, along with the statistical level of significance are show in Table 3. Polymorphisms were observed for all SNPs but in the case of the SOD2 Ile58Thr polymorphism, only dominant homozygotes (Thr/Thr) were found. A significant difference in genotype frequency between the cohorts was only observed for the CAT -262C>T SNP. The CC genotype was less frequent in the donor cohort (50%) compared to that of the patient group (69%), while the frequency of the CT genotype was almost double in the donor cohort (47%) compared to that found in the patient cohort (26%) (P = 0.002).

The odds ratio (OR) of being infertile for each genotype was calculated against the other two genotypic options. A significant OR was only noted in catalase CC and CT genotypes (Figure 1); in fact, the CC genotype was associated with a 2 fold increased risk of infertility (OR = 2.262; 95% CI = 1.369 - 3.733; P = 0.001), while the CT genotype was associated with a protective effect (OR = 0.401; 95% CI = 0.241 - 0.667; P = 0.001).

Median differences in classical seminal parameters (seminal volume, sperm concentration, % sperm motility, % sperm morphology and % SDF) were compared between patients and donors for each of the genotypes (Table 4). The results of the analysis revealed significant differences for only the catalase SNP with respect to motility (P = 0.047) and morphology (P = 0.024). Pairwise comparisons for these parameters also revealed significant differences for morphology between the CC and CT genotypes (H = -32.187, adjusted P = 0.029, r = -0.130). Moreover, analysis of the median values for sperm concentration between patients and donors for the SNP CAT C-262T genotypes were close to being significant (P = 0.051).

Discussion

This study explored the association of 6 polymorphisms of antioxidant enzymes (SOD2.lle58Thr, SOD2.rs4880, CAT C-262T, GPX1 Pro200Leu, GSTT and GSTM) with observed infertility in patients from a Spanish fertility clinic; a statistically significant association was observed between the SNP for CAT C-262T and infertility. The CT genotype was associated with a protective or beneficial effect with respect to fertility.

For the purposes of this study we have assumed that donors and patients attending the clinic were representative of the current Spanish cosmopolitan population as the clinic is located in Madrid (Spain); we therefore compared the genotype frequencies of the Madrid population with that obtained for the same polymorphisms in other European populations (Table 5) [13, 17]. We observed that for SNP GPX Pro200Leu and for the variants of the GST enzymes our frequencies were within or close to within the frequency limits obtained for the same polymorphisms of other European populations. For the SNP SOD rs4880 the only differing frequency was for the heterozygote genotype (0.56 vs 0.28 to 0.47). In contrast, the SNP CAT C-262T was the one of the largest differences for all genotype options, while for SNP SOD2 Ile58Thr, we could only find the dominant homozygote genotype in the current population.

The catalase enzyme (CAT), coded in chromosome 11p3, is the main enzyme in the detoxification of H_2O_2 to H_2O. H_2O_2 plays an important role in male infertility as it causes lipid peroxidation, which reduces the fluidity of the membrane, thus causing a reduction in motility and poor oocyte-sperm membrane fusion [18, 19]. H_2O_2 in seminal plasma can appear directly as a consequence of the activity of leukocytes or indirectly as a product of the detoxification of ROS by superoxide dismutase. It has been shown that seminal plasma of infertile patients has a higher H_2O_2 concentration and a lower catalase activity than that of healthy controls [20].

Recent studies have also demonstrated that an external supplementation with catalase when cryopreserving semen improves post-thaw sperm motility and sperm viability [21].

In the current study we found significant differences in the genotype frequencies for SNP CAT C-262T between patients and fertile donors. Specifically, we observed an increased frequency (almost double) of the CT genotype in donors when compared with patients. Thus, we hypothesise that the T allele may be a variant of the enzyme with a higher transcriptional rate and be beneficial in heterozygosity. This observation is concordant with the studies of Sabouhi et al. [12] and Foresberg et al. [22], both of whom observed the T allele to be related to higher catalase levels. In contrast, Tefik et al. [11] and Ahn et al. [23] showed a lower enzyme activity for the CT and TT genotypes of the CAT C-262T polymorphism; it is possible that these differences may be a consequence of the particular characteristics of the populations included in each study. According to Suzan et al. [13] the CAT C-262T polymorphism shows important differences in the genotype frequencies between populations. Moreover, when we compared the values of the main parameters of the traditional sperm analysis between genotypes we observed that the highest quality values for all parameters appeared in the genotypes containing the T allele. Significant differences between genotypes were also obtained for motility and morphology, although pairwise comparisons only revealed significant differences for morphology between the CT and CC genotypes, with CT genotype being associated with better semen quality; close to significant differences were also apparent for the sperm concentration parameter. Moreover, for sperm DNA fragmentation, the highest sperm quality was also found associated with the CT genotype. Finally, it was noted that the occurrence of infertility was 2.26 times more likely to be associated with the CC genotype, than for the other 2 genotypes, while the CT genotype showed a protective effect; these observations support the hypothesis that the CT genotype could be beneficial in terms of protection against oxidative stress.

Manganese superoxide dismutase 2 (SOD2) is a mitochondrial antioxidant enzyme coded in chromosome 6q25. It catalyses the detoxification of superoxide radicals in the mitochondrion producing H_2O_2 and O_2 as by-products. In the SOD2 rs4880 polymorphism there is a change of Ala to Val in position -9 of the N-terminal mitochondrial targeting sequence which leads to a variant of the enzyme that is less efficiently transported to the mitochondrion [24]. The results of our study were not consistent with this hypothesis as we observed a slightly higher representation of the Val allele in fertile donors than in patients. However, our results are concordant with the observations of Yan et al. [10] that found a higher presence of the Val allele in fertile males; they suggested that the higher efficient Ala allele should produce higher levels of H_2O_2 which may be detrimental for sperm quality. Moreover, we noted that Ala/Val and Val/Val genotypes were associated with higher quality seminal parameters, although the differences between these genotypes were low; in addition, the Ala/Ala genotype showed a 1.6 times higher odds ratio of being infertile than the other two genotypes.

The Sod2 Ile58Thr polymorphism affects the stability of the protein [11]. In our study we could not find a representation of that polymorphism in our population as we only found the Ile/Ile genotype both in patients and in donors; this observation would suggest that the Ile/Ile genotype is over-represented in our studied population.

The glutathione peroxidase 1 enzyme (GPX1), coded in chromosome 3p21, is a selenium dependent peroxidase and is ubiquitously expressed in humans. It works as the final electron transporter that neutralizes peroxide radicals to water and oxygen [25]. In the Pro200Leu polymorphism, there is a substitution of Pro to Leu at codon 200 located in the c-terminal region of the protein. Najafi et al. [26] in a computer predictor study concluded that this polymorphism is located on the protein surface in a non-functional region and that the substitution does not affect protein stability or structure. However, Hu et al. [27] concluded that

the Leu allele is less effective than the Pro allele when neutralizing peroxide radicals; in contrast with this observation, our results showed a higher presence of the Leu allele in fertile donors than in patients and better values for almost all the parameters in the traditional sperm analysis in the Leu/Leu genotype group (the only parameter differing was the volume). Curiously, we also found significant differences between genotypes for the parameter volume, both in the patient population and in the global analysis. The odds ratio analysis for infertility showed that the ratio of being infertile was almost similar for the Pro/Leu and Leu/Leu genotypes and was 1.27 times higher for the Pro/Pro genotype than for the other two.

Glutathione S transferase (GST) comprises a family of enzymes which conjugate toxic intermediates with glutathione inactivating them and facilitating their excretion. GSTM (chromosome 1p13.3) and GSTT (chromosome 22q11.2) are two human GST isozymes which can present a null allele as a result of a deletion. Several studies have been carried out recently to try and clarify the relationship between the null variants and male infertility but this relationship is still controversial. For the GSTM null genotype, some studies have concluded that it is associated with a higher risk of male infertility [28, 29] but others show no relationship at all [14]. Similarly, although several studies have shown a relationship between the GSTT null genotype and male infertility [30] there are others that reveal no relationship or even a beneficial effect of the null genotype with respect to male infertility [14]. Moreover, some studies point out that the effects of both null genotypes may also be related to exposure to air pollution [31]. In our study, we observed almost similar frequencies and an odds ratio of infertility for both patients and donors with respect GSTT, whereas the GSTM null genotype showed a slightly higher frequency in patients than in donors and an odds ratio indicating that the occurrence of infertility was 1.25 times more likely than for the wild variant. Consequently, our findings are concordant with the most accepted hypothesis that both null variants are detrimental for male infertility.

We are aware that our study has both strengths and weaknesses. The first strength is the relatively high number of individuals included in the study; second, all subjects included in the donors' cohort had proven fertility as they had already fathered at least one child. With respect to the study's weaknesses, althoughwe knowthat there is a difference in themean age of the two cohorts included in the study, all males included in the study were young men within the well-recognized fertility window and we are confident that this difference is not confounding our results as age does not influence genotypic frequencies. In addition, although seminal parameters are influenced by patient exposure factors such as BMI, smoking, and diet, these aspects are difficult or virtually impossible to fully define in the individual patients and thus we have not considered them in our study.

In conclusion, we found statistically significant differences for SNP CAT C-262T between fertile donors and infertile patients, with the CT genotype showing a potentially protective role. However, we also found interesting associations for the other polymorphisms we examined that could help us to understand the roles of the antioxidant pathways in ROS related male infertility. According to our observations, we predict that males with the Val allele in SOD gene, the Leu allele in GPX gene and GSTs with wild variants should have a better response to increased ROS. We are confident that more research as to the relationship between gene polymorphisms associated with seminal plasma antioxidant capacity will lead to a better understanding of male factor infertility and could therefore be applied to improve both patient diagnosis and donor selection with genetic background testing.

5. References

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Table 1. Primers and restriction enzymes used in the PCR-RFLP study.

SNP	Primer PCR	Annealing Tº	PCR product	Rest. enzyme	Restriction fragments
SOD2 Ile58Thr	F 5'-AGCTGGTCCCATTATCTAATAG-3' R 5'-TCAGTGCAGGCTGAAGAGAT-3'	56 ºC	140 bp	EcoRV	Thr/Thr 117 Ile/Thr 140,117 Ile/Ile 140
SOD2 RS 4880	F 5'-GCCCAGCCTGCGTAGACGGTCCC-3' R 5'-TGCCTGGAGCCCAGATACCCCAAG-3'	59 ºC	110 bp	HaeIII	Ala/Ala 23,85 Ala/Val 23,85,110 Val/Val 110
CAT -262 C→T	F 5'-ATTCCGTCTGCAAAACTGGC-3' R 5'-GAGCCTCGCCCCGCCGGCCCG-3'	59 ºC	129 bp	SmaI	CC 99 CT 109,99 TT 109
GXP1 Pro200Leu	F 5'-TGTGCCCCTACGCAGGTACA-3' R 5'-CCAAATGACAATGACACAGG-3'	57 ºC	338 bp	HaeIII	Pro/Pro 251,81 Pro/Leu 338,251,81 Leu/Leu 338

Table 2. Demographic and clinical profile of the participant subjects (Mean ± Standard deviation). *P*-value calculated with the Mann-Whitney U-test.

	Patients	Donors	*P*-value
Age (year)	37.81 ± 5.05	24.76 ± 4.96	<0.001
Volume (mL)	3.33 ± 1.36	3.19 ± 1.19	0.431
Sperm Concentration (X 10^6 mL^{-1})	26.93 ± 18.41	53.82 ± 18.92	<0.001
Motility (%)	38.93 ± 12.22	54.55 ± 6.36	<0.001
Morphology (%)	3.41 ± 1.87	6.41 ± 1.71	<0.001
DNA fragmentation (%)	19.69 ± 13.15	No data	No data

Table 3. SNP Genotype frequencies of patients and donors. *P*-value calculated by chi-square test.

Genotype	Patients n (obs)	Genotype frequency	Donors n (obs)	Genotype frequency	*P*-value
Sod2 Ile58Thr					
Thr/Thr	313	1.00	80	1.00	
Ile/Thr	0	0.00	0	0.00	0
Ile/Ile	0	0.00	0	0.00	
Sod2 rs4880					
Ala/Ala	69	0.22	12	0.15	
Ala/Val	150	0.48	45	0.56	0.329
Val/Val	94	0.30	23	0.29	
CAT -262 C>T					
CC	215	0.69	40	0.50	
CT	82	0.26	37	0.47	0.002
TT	16	0.05	3	0.03	
GPX1 Pro200Leu					
Pro/Pro	139	0.44	31	0.39	
Pro/Leu	138	0.44	38	0.47	0.669
Leu/Leu	16	0.12	11	0.14	
GSTT					
Present	246	0.79	63	0.78	
Absent	67	0.21	17	0.22	0.975
GSTM					
Present	151	0.48	43	0.53	
Absent	162	0.52	37	0.47	0.420

Figure 1: Forest plot of infertility risk for each genotype. Horizontal lines represent 95% CI. Each square represents the OR point. The vertical line is at the null value (OR=1). *P*-value calculated by chi-square test.

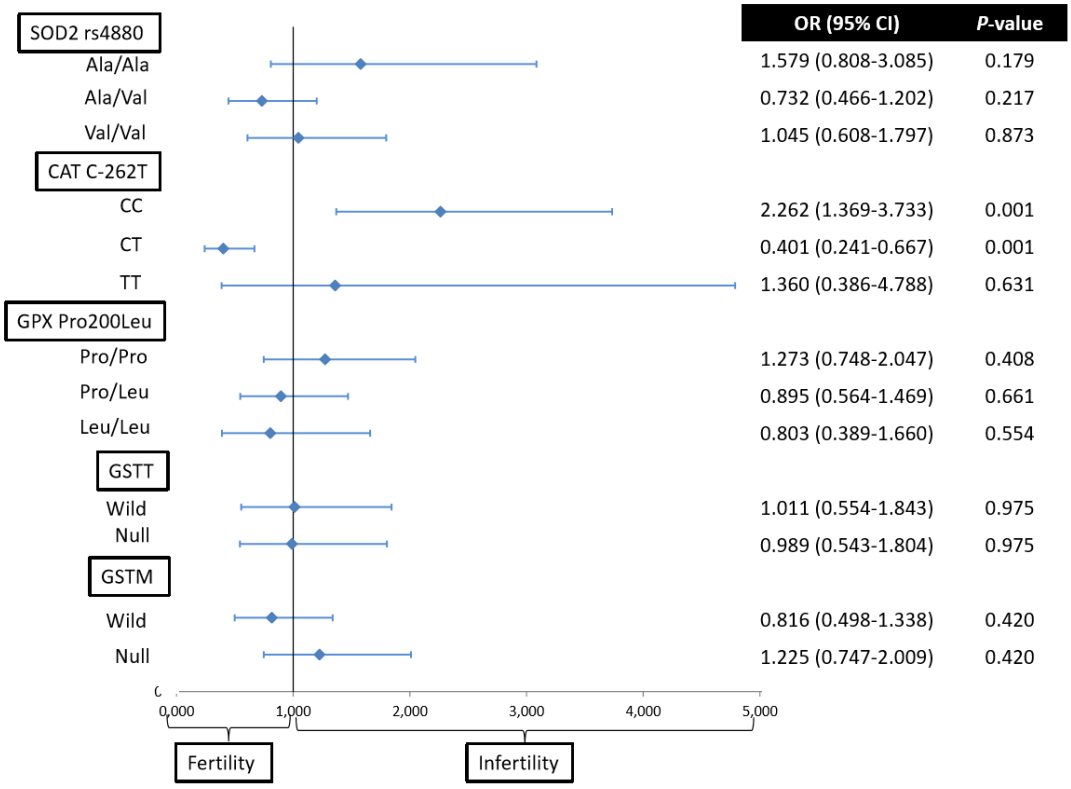

Table 4. Extended analysis of traditional seminal parameters for genotypes comparing patients versus donors. The data shown are the mean for volume (mL), concentration (10^6 mL^{-1}), motility (%), morphology (%), DNA fragmentation (%). *P*-value$_1$ = comparison between genotypes from patients and donors, *P*-value$_2$ = comparison between genotypes for all 393 samples. Statistical analysis - the Kruskal-Wallis test was used to compare for volume, sperm % motility, %morphology and % DNA fragmentation for the 3 genotypes, whereas, the Mann-Whitney U-test was used to analyze data when 2 genotypes were compared.

			Genotypes			P-value$_1$	P-value$_2$
SOD2rs4880			Ala/Ala	Ala/Val	Val/Val		
	Volume	Patients	3.25	3.43	3.21	0.749	0.781
		Donors	3.08	3.27	3.08	0.722	
	Concentration	Patients	26.19	26.83	27.47	0.983	0.736
		Donors	51.50	54.56	53.60	0.984	
	Motility	Patients	37.04	39.85	38.54	0.354	0.108
		Donors	55.83	54.77	53.47	0.567	
	Morphology	Patients	3.34	3.43	3.39	0.955	0.526
		Donors	6.41	6.59	6.04	0.768	
	DNA fragmentation	Patients	21.06	19.31	19.32	0.634	0.634
		Donors					
CAT -262 C>T			CC	CT	TT		
	Volume	Patients	3.37	3.16	3.53	0.504	0.820
		Donors	3.07	3.36	2.60	0.487	
	Concentration	Patients	26.26	27.51	33.02	0.419	0.051
		Donors	52.93	53.94	64.00	0.294	
	Motility	Patients	38.48	38.72	45.93	0.080	0.047
		Donors	55.25	54.18	50.00	0.171	
	Morphology	Patients	3.31	3.54	4.12	0.159	0.024
		Donors	6.48	6.48	5.00	0.088	
	DNA fragmentation	Patients	19.84	18.66	22.88	0.849	0.849
		Donors					
GPX1 Pro200Leu			Pro/Pro	Pro/Leu	Leu/Leu		
	Volume	Patients	3.31	3.49	2.79	0.018	0.044
		Donors	3.20	3.20	3.11	0.983	
	Concentration	Patients	27.92	25.37	29.05	0.780	0.701
		Donors	53.19	54.05	54.81	0.789	

	Motility	Patients	38.64	38.88	39.44	0.830	0.783
		Donors	55.64	54.05	53.18	0.642	
	Morphology	Patients	3.41	3.31	3.75	0.597	0.618
		Donors	6.25	6.59	6.18	0.866	
	DNA fragmentation	Patients	20.88	19.06	17.64	0.509	0.509
		Donors					
GSTT			Wild (+)		Nul (-)		
	Volume	Patients	3.33		3.33	0.651	0.889
		Donors	3.25		2.95	0.508	
	Concentration	Patients	27.50		24.85	0.569	0.604
		Donors	53.46		58.76	0.620	
	Motility	Patients	39.00		38.65	0.921	0.859
		Donors	54.91		54.91	0.340	
	Morphology	Patients	3.42		3.35	0.855	0.986
		Donors	6.21		6.21	0.460	
	DNA fragmentation	Patients	19.65		19.82	0.923	0.923
		Donors					
GSTM			Wild (+)		Null (-)		
	Volume	Patients	3.32		3.34	0.464	0.719
		Donors	3.30		3.05	0.460	
	Concentration	Patients	27.25		26.64	0.704	0.460
		Donors	53.14		54.59	0.961	
	Motility	Patients	39.93		38.00	0.365	0.266
		Donors	53.81		55.40	0.152	
	Morphology	Patients	3.46		3.36	0.455	0.256
		Donors	6.54		6.24	0.556	
	DNA fragmentation	Patients	19.33		20.03	0.819	0.819
		Donors					

Table 5: Frequencies obtained for the polymorphisms in other studies with European population. Adapted from Suzen et al. [13] and Xiong et al. [17]

	Dominant homozygote	Heterozygote	Recessive homozygote
SOD2 Ile58Thr	0.65	0.32	0.02
SOD2 rs4880	0.06-0.054	0.28-0.47	0.08-0.52
CAT C-262T	0.57-0.63	0.30-0.37	0.03-0.05
GPX Pro200Leu	0.41-0.59	0.35-0.45	0.06-0.15
GSTT			0.13-0.26
GSTM			0.42-0.60