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Estrogen Receptor alpha and beta Polymorphisms Are Associated with Semen Quality

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ABSTRACT

The role of estrogen receptor α and estrogen receptor β gene polymorphisms on semen quality is the aim of our study. One hundred fourteen men were examined in the IVF Unit and it was found that 85 men had normal sperm count and 29 were oligozoospermic. The genotype analysis, on DNA extracted from spermatozoa, revealed that in men with oligozoospermia (sperm concentration $<20x10^6$ spermatozoa/ml), those with ERα 397T/C and 397C/C genotypes had higher sperm motility while those with 397T/T genotype had lower sperm motility (p=0.003). In addition, men with ERα 351A/A genotype had lower sperm motility compared with 351A/G and 351G/G genotypes (p=0.013). Furthermore, normal sperm count men with ERα 397T/T genotype had higher sperm concentration compared with 397T/C and 397C/C genotypes (p=0.016), while men with ERα 351A/A genotype had higher sperm concentration from those with 351A/G and 351G/G genotypes (p=0.05). In contrast, no significant associations were found between ERβ (1082G→A & 1730A→G) polymorphisms and sperm concentration or motility. In conclusion, ERα polymorphisms were found to be associated with sperm motility and concentration supporting the significance of this gene in spermatogenesis and semen quality.

Key Words: *sperm, estrogens, infertility, estrogen receptor*

INTRODUCTION

Estrogens play an important role in the differentiation, maturation and function of the human reproductive system. In recent years, considerable emphasis has been focused on the role of estrogens in the regulation of male reproduction. Human and animal models have evidenced an association between estrogen insufficiency with abnormal spermatogenesis and male infertility (O'Donnell *et al.*, 2001, Hess *et al.*, 2003). Estrogens are synthesized in the male reproductive system from testosterone via the action of aromatase cytochrome P450 (Nitta *et al.*, 1993) by at least three different cell types: Sertoli cells, Leydig cells, and germ cells (Carreau *et al.*, 2003, Bourguida *et al.*, 2003) implicating an important role in testicular function. It has been reported that estrogens reduce testosterone production from Leydig cells (Zhai *et al.*, 1996), reduce Sertoli cell numbers in adult when they are given during development (Atanassova *et al.*, 1999), disrupt fetal Leydig cells development (Delbes *et al.,* 2005), inhibit apoptosis of human post- meiotic germ cells (Pentiainen *et al.*, 2000), and increase spermatogonial number per testis (Gould *et al.*, 2007).

Estrogen action is mainly mediated by two specific receptors, ERα and ERβ, which are found within the testis. These receptors are highly homologous ligand-inducible transcription factors and can regulate the expression of specific genes. The central role of estrogens in the male reproductive system has focused attention on the presence and distribution of their cognate receptors in male reproductive tissues and cells. Both receptors are expressing in human testicular germ cells in different stages of spermatogenesis from spermatogonia to elongated spermatids (O'Donnell *et al.,* 2001). ERα was found in primary and secondary spermatocytes and elongated spermatids (Pentikainen *et al.,* 2000), whereas, ERβ

expression appears to be the predominant ER in spermatogonia (O'Donnell *et al.,* 2001). It was found also in pachytene spermatocytes and spermatids (Pentikainen *et al.,* 2000; Makinen *et al.,* 2001; Saunders *et al.,* 2001).

Human ejaculated spermatozoa express both receptors (ERα and ERβ) (Aquila *et al.,* 2004). However, conflicting data exist regarding their localization. Immunolocalization experiments have demonstrated that ERα was prevalently localized in the midpiece region, whereas, ERβ was detected in the tail region, with an overlapping distribution of both receptors in the proximal region of the tail (Aquila *et al.,* 2004). On the other hand, Solakidi and co-workers (2005) have demonstrated that most of the ERα immunostaining has been in the form of a compact zone at a region corresponding to the equatorial segment of the upper post-acrosomal region of the sperm head, whereas most of the ERβ immunostaining has been in the midpiece, at the site of the mitochondria, suggesting distinct roles of these receptors in the physiology of sperm cells and in the process of fertilization. Despite discrepancies regarding their localization the expression of both ERs in the midpiece and the tail of human spermatozoa suggest that these receptors could be involved in the mitochondrial function affecting the motility of spermatozoa.

Polymorphic variants of both ERα and ERβ genes have been identified in recent years and studied for possible association with reproductive and other clinical outcomes (Georgiou *et al.*, 1999, Syrrou *et al.*, 1999, Weel *et al.*, 1999). A naturally occurring mutation (replacement of cytosine with thymidine at codon 157) in ERα has been found to influence sperm viability but not sperm density increasing gonadotropin and estradiol levels (Smith *et al.,* 1994). Two polymorphisms in ERα (*XbaI* and *Pvull*) have been associated with azoospermia or severe oligozoospermia (Kukuvitis *et al.*, 2002, Suzuki *et al.*, 2002). A potential role of the multiallele (TA)n polymorphism in the sperm production in infertile men have been also described by Guarducci and co-workers (2006). In case of ERβ, an association of the *RsaI* polymorphism with male infertility has been found, probably due to effects on LH secretion (Aschim *et al.*, 2005). These findings are implying that genetic polymorphisms of ERs genes might modify sperm characteristics.

In the present study, we sought to investigate the role and the possible synergism of the ERα *PvuII* (397T→C) and *XbaI* (351A→G) polymorphisms and ERβ *RsaI* (1082G→A) and *AluI* (1730A→G) polymorphisms on sperm concentration and motility. In the international literature there is no other study associating both ERα and ERβ polymorphisms with male infertility.

MATERIALS AND METHODS

Subjects

The study population consisted of 114 men who were referred to the IVF Unit of the Department of Obstetrics and Gynecology, Medical School of Ioannina, Greece, for andrological examination. A complete medical history was taken and physical examination was performed. Semen analysis was performed according to World Health Organization (WHO, 1999) guidelines: sperm concentration>20x10^6/ml, motility grade A+B>50% and, >14% normal forms (strict criteria, Kruger *et al.*, 1987). Men were asked to abstain from sexual activity for 48 h to 5 days prior to the investigation. Values for semen parameters were calculated as means of two analyses taken at least 1 month apart. Men suffering from hypogonadotropic hypogonadism, or obstructive syndromes of the seminal tract, carrying microdeletions of the long arm of the Y chromosome or with karyotypic abnormalities and men under treatment with spermatogenesis-impairing medication were excluded from the current study. The Institutional Ethics Committee approved the study protocol in accordance to the Helsinki declaration and all participants gave informed consent. Sperm analysis and DNA analysis were done by two independent investigators who were blind to the results of the other party.

DNA Extraction

DNA was extracted from sperm according to a protocol previously described (Lazaros *et al.*, 2008a). In brief, approximately 7x10^6 spermatozoa were mixed with 1xPBS (Invitrogen, Carlsbad, California) and centrifuged at 3,000 rpm for 6 min. The supernatant was discarded and the precipitate was diluted in 200 μL 1xPBS and centrifuged at 10,000 rpm for 1 sec. Subsequently, 200 μL of 1xPBS, 15 μL SDS 10% (Invitrogen, Carlsbad, California), 13 μL DTT (Sigma-Aldrich Company LTD, United Kingdom) and 0.2 mg proteinase K (Macherey-Nagel, Duren, Germany) were added and the mix was incubated for 2 hours. Then, 85 μL 6M NaCl (MERCK, Darmstadt, Germany) were added and the dilution was mixed and centrifuged at 12,000 rpm for 3.5 min. Finally, the supernatant was transferred into a clean tube and 750 μL of frozen absolute ethanol were added. The isolated DNA was preserved in 50 μL TE (Invitrogen, Carlsbad, California) at 4^oC.

Genotype analysis

The amplification of the estrogen receptor alpha polymorphic sites 397T→C and 351A→G, which are located 397 and 351bp, respectively, upstream from the first nucleotide of exon 2 of ERα, has been described previously (Lazaros *et al.*, 2008b). The genotypes for *PvuII* (397T→C) and *XbaI* (351A→G) polymorphic sites were characterized as TT/TC/CC and AA/AG/GG, respectively.

Genotyping for the 1082G→A and 1730A→G polymorphic variants of the ERβ gene was carried out as follows. The ligand binding domain of exon 5 and the 3'-untranslated region of exon 8 of the ESRβ gene were amplified using the following primer pairs: 5'-TCTTGCTTTCCCCAGGCTTT-3', 5'-ACCTGTCCAGAA CAAGATCT-3' and 5'-GACCTGCTGCTGGAGATGCT-3', 5'-AAT GAGGGACCACACAGCA-3', respectively. PCR amplification was performed in a total volume of 25 μL consisting of 1 μL extracted DNA, 0.2 mM dNTPs (Invitrogen, Carlsbad, California), 0.8 μM of each primer, 1x Taq DNA polymerase buffer, 1mM $MgCl_2$ and 1U of Taq DNA polymerase (Invitrogen, Carlsbad, California). The thermal cycling was as follows: denaturation at 95 °C for 2 min, 30 cycles of 94 °C for 1 min, 62 °C for 1 min, 72 °C for 1,30 min with a final extension at 72 °C for 10 min. PCR products were subsequently digested with restriction enzymes *RsaI* and *AluI* (Invitrogen, Carlsbad, California), respectively. Enzyme digestion products were separated by 2% agarose gel electrophoresis and visualized by exposure to ultraviolet light after ethidiun bromide staining. The resulting genotypes for *RsaI* (1082G→A) and *AluI* (1730 A→G) polymorphic sites were characterized as GG/GA/AA and AA/AG/GG, respectively, and diplotypes were also recorded, matching each genotype of *RsaI* (1082G→A) polymorphism with each genotype of *AluI* (1730A→G) polymorphism. All samples were turn in duplicates with negative and positive controls for each of the three genotypes expected and blanks.

Statistical analysis

Statistical analysis of differences in allele and genotype frequencies was performed using the chi-square test. Normal distribution of continuous parameters was tested by Kolmogorov-Smirnov test. Differences in continuous parameters were assessed by using t-test for independent variables or the non-parametric Mann-Whitney U test and Kruskal-Wallis test as appropriate. Pearson's coefficient of correlation was used for bivariate regression analysis. P-value of < 0.05 was set as statistically significant. All results are reported as the mean ± SD. All analyses used the SPSS statistical package (version 14.0, SPSS Inc, Chicago, IL, USA).

RESULTS

Study population characteristics

The study population comprised by 114 men aged 33.2±7.5 years. This group was consisted of 85 men with normal sperm count (84.2±63.5 x10^6 sperm/ml) and 29 oligozoospermic patients (8.3±5.4 x10^6 sperm/ml). Men with normal sperm count were further subdivided in those (n=49) with normal sperm motility (63.9±9.5%) and those (n=36) with low sperm motility (35.6±9.1%).

All polymorphisms were in Hardy Weinberg equilibrium in normal sperm count men and oligospermic patients.

Genotype distribution

The genotype frequencies of normozoospermic and oligozoospermic men are presented in Table 1. The allele distribution, allele frequencies and genotypes were not significantly different between men with normal sperm count and oligozoospermic men.

When the study population divided according to the motility of spermatozoa (normal motility grade A+B> 50%; asthenozoospermia grade A+B< 50%), although 351A/G, 397T/C, 1730A/G and 1082G/A genotypes appeared with higher frequency in asthenozoospermic men comparing with men with normal motility, there were no statistical significant differences between the two groups (Table 1).

Association of ERα and ERβ polymorphisms with sperm characteristics

For the analysis of the effect of the estrogen receptors polymorphisms on sperm motility, the study population was subdivided in two subgroups, namely men with sperm concentration $<20x10^6$ spermatozoa/ml and men with sperm concentration $>20x10^6$ spermatozoa/ml (Table 2).

ERα polymorphisms and sperm motility

In oligozoospermic men, an association was found between *PvuII* and *XbaI* genotypes with sperm motility. In case of *PvuII*, there was a significant difference in the distribution of the polymorphism between men with high sperm motility versus low sperm motility (p=0.013) (Table 2). Especially men with 397T/C and 397C/C genotypes had higher sperm motility while those with 397T/T genotype had lower sperm motility (p=0.003) (Figure 1). Namely, 397C allele carriers presented increased sperm motility. In case of *XbaI,* there was also a significant difference in the distribution of the polymorphism between men with high sperm motility and men with low sperm motility (p=0.045) (Table 2). Especially, men with 351A/G and 351G/G genotypes had higher sperm motility while those with 351A/A genotype had lower sperm motility (p=0.013) (Figure 1). Namely, 351G allele carriers presented increased sperm motility. However, these associations were not appeared in men with sperm concentration $>20x10^6$ spermatozoa/ml.

ERα polymorphisms and sperm concentration

In the group of normal sperm count men, the genotype analysis revealed a strong association between ERα 397T→C and 351A→G polymorphisms and sperm concentration. In case of *PvuII*, men with 397T/T genotype had higher sperm concentration while those with 397T/C and 397C/C genotypes

had lower sperm concentration (p=0.016) (Figure 2). Furthermore, in case of *XbaI,* men with 351A/A genotype had higher sperm concentration while those with 351A/G and 351G/G genotypes had lower sperm concentration (p=0.05) (Figure 3). However, no significant association was found between these polymorphisms and sperm concentration in oligospermic patients.

ERβ polymorphisms and sperm characteristics

In the case of the ERβ *AluI* (1730 A→G) polymorphism, there was no statistical significant difference in the distribution comparing men with normal sperm count and oligospermic patients. In the total study population, men with 1730G/G genotype had higher sperm motility compared with men with 1730A/Aand 1730A/G genotypes (45.6±17.2 vs 53.2±20.9, p=0.044). In contrast, no significant association was found between *AluI* (1730 A→G) polymorphism and sperm concentration.

When we analyzed the ERβ *RsaI* (1082G→A) polymorphism distribution, no significant association was found with sperm concentration or sperm motility, probably due to the low frequency of the polymorphism.

DISCUSSION

Over the last two decades a growing body of evidence has been accumulated suggesting a pivotal role of estrogens in male reproduction. In order to exert a biological role, testicular estrogens should interact with estrogen receptors (ERs) which in turn modulate the transcription of specific genes. Our data indicate that functional polymorphisms of ERs can influence human spermatogenesis, alone or in combination with a specific genetic background or certain environmental factors (Krausz *et al.*, 2001).

In the current study, we investigated the effect of four functional polymorphisms, the ERα 397T→C and 351A→G polymorphisms and the ERβ 1730A→G and 1082G→A polymorphisms on sperm concentration and motility. These polymorphic variants are known to influence estrogen levels through mediation by two specific receptors affecting the fertilizing ability of spermatozoa (Kukuvitis *et al.*, 2002, Aschim *et al.*, 2005, Su *et. al.,* 2008). These observations are showing the association of ERs polymorphisms with male infertility.

The importance of ERs in human spermatogenesis has been elucidated also after studies on genetically modified mice lacking functional ERα (αERKO) or ERβ (βERKO). Eddy and co-workers (1996) showed that the targeted disruption of the ERα causes alteration of spermatogenesis, reduced motility of spermatozoa and infertility. In addition, knockout and double knockout mice for ERs are infertile since puberty and the phenotype of testicular tissue showed atrophy of the testes and seminiferous tubule dysmorphogenesis, which in turn, resulted in decreased spermatogenesis and sperm motility (Krege *et al.,* 1998, Eddy *et al.,* 1998, Couse *et al.,* 1999, Gould *et al.,* 2007). The 397T→C and

351A→G polymorphisms probably affect the ERα function, influence estrogen levels and alter sperm motility in men with low sperm concentration.

How ERα polymorphism can alter the motility of spermatozoa? In the current study, regarding the ERα gene, an association was found between 397T→C (*PvuII*) and 351A→G (*XbaI*) polymorphisms with sperm motility only in men with sperm concentration $<20x10^6$ spermatozoa/ml. In particular, men with 397T/C, 397C/C and 351A/G, 351G/G genotypes had higher sperm motility compared with those with 397T/T and 351A/A genotypes, respectively. Namely, in carriers of 397C and 351G alleles a noteworthy correlation with high sperm motility was found. The existence of ERs on the sperm membrane (Luconi *et al.,* 1999) and in the midpiece of spermatozoa (Aquila *et al.,* 2004; Solakidi *et al.,* 2005) indicates a role of estrogens in male gamete motility (Carreau *et al.,* 2007a). Recent data have indicated a mechanism by which estrogens can regulate mitochondrial function by increasing nuclear respiratory factor-1 (NRF-1) expression (Mattingly et al., 2008). Specifically, estradiol stimulates mitochondrial function through a genomic mechanism of ER action involving direct ERα and ERβ interaction with a nonconsensus estrogen response element (ERE) in the NRF-1 promoter. In vivo knockdown experiments have indicated that estradiol stimulates NRF-1 transcription and consequently increase mitochondrial biogenesis through ERα activity and not through ERβ activity at least in MCF-7 breast cells (Mattingly *et al.,* 2008). The latter data allowing the hypothesis that ERα polymorphisms can increase mitochondrial activity via NRF-1 transcription in human ejaculated spermatozoa presenting them with high motility.

Are there ERα in the midpiece region of human ejaculated spermatozoa where mitochondria are occurring? Various immunolocalization studies are giving different results. According to Durkee and co-workers (1998) and Aquila and co-workers (2004) ERα was immunolocalized prevalently in the midpiece region of spermatozoa. On the other hand, Solakidi and co-workers (2005) were found only ERβ and not ERα staining together with the mitochondria in the midpiece. Despite the fact that different isoforms of ERs can be localized in the midpiece region, all authors agreed that this discrepancy in the localization of ERα attributed to the different processing methods and the use of a different anti-ERα antibody. According to the latter data, one can assume that both receptors are localized in the midpiece region of spermatozoa and their detection is mainly depending of the anti-ER antibody used. However, more studies are needed to verify this theory.

In mouse as well as in man it has been shown that estrogens are positively involved in sperm capacitation and acrosome reaction (Carreau *et al.*, 2007b). The existence of ERα at the upper post-acrosomal sperm head region and the presence of ERβ at the midpiece, at the site of mitochondria (Solakidi *et al.*, 2005) is likely to be relevant for a role of estrogens in male gamete maturation and motility. It is possible that estrogens produced locally should be considered as a physiologically relevant

hormone involved in the regulation of sperm motility. ERα polymorphisms may influence these locally acting estrogen levels (Carreau *et al.*, 2007a), with effects on sperm motility in men with low sperm concentration. In normal sperm count men, ERα polymorphisms may influence sperm concentration, either on spermatogenesis directly, or through serum estrogen levels.

In the current study, an association was found between 397T→C (*PvuII*) and 351A→G (*XbaI*) polymorphisms with sperm concentration in the normal sperm count men. Specifically, men with 397T/T and 351A/A genotypes had higher sperm concentration while those with 397T/C, 397C/C and 351A/G, 351G/G genotypes had lower sperm concentration, respectively. This finding is in accordance with a previously described association of ERα polymorphisms with male infertility (Kukuvitis *et al.,* 2002). In that study, specific genotypes of the *XbaI* polymorphism was correlated well with azoospermia or idiopathic severe oligospermia showing that XbaI polymorphism can affect the concentration of spermatozoa. In contrast, similar association was not found for the PvuII polymorphism in the study by Kukuvitis and co-workers (2002) possibly because of the frequency differences of the two ERα polymorphisms in the group with men with normal sperm count and the different study population. The current study does not include azoospermic and severe oligozoospermic men.

Although the role of ERα in human spermatogenesis is still under investigation, a human equivalent of the αERKO mice have shown reduced number of spermatogonia, spermatocytes and spermatids per testis as well as increased germ cell apoptosis (Gould *et al.,* 2007). Since estrogen regulates the reabsorption of luminal fluid in the head of the epididymis, reduced sperm production is thought to be a consequence of impaired fluid resorption within the efferent ducts of the testis (Eddy *et al.*, 1996). Taking into account the above results of ERα inactivation, we can hypothesize that 397C and 351G alleles probably reduce ERa function and simultaneously sperm concentration.

Human testis has two main functions: production of spermatozoa and steroids synthesis. Estrogens are produced from the transformation of androgens by aromatase. In humans, biologically active aromatase and ERs (α and ß) were present in ejaculated spermatozoa with excess residual cytoplasm (Rago *et al.*,2006) and in immature germ cells (Lambard *et al.*,2003) in addition to Leydig cells. Careau and co-workers (2007a) have demonstrated that the amount of P450 aromatase transcripts is 30% lower in immotile than in motile spermatozoa. Moreover, the aromatase activity was 50% greater in motile fraction compared to immotile spermatozoa. The observations of i) decreased sperm motility in men with aromatase deficiency and ii) decrease of aromatase in immotile human spermatozoa, could suggest that aromatase is involved in the acquisition of sperm motility.

In the case of the ERβ *AluI* (1730 A→G) polymorphism, all men from the study population with 1730G/G genotype had higher sperm motility compared with men with 1730A/A and 1730A/G genotype. However, we did not find any significant difference between infertile men and controls. The same was

observed by Aschim and co-workers (2005), who studied infertile men with sperm concentration lower than $5x10^6$ spermatozoa/ml. Our finding that men with *AluI* AA and AG genotypes presented with decreased sperm motility may indicate decreased ERβ function. Taking into account that i) ERβ was significantly associated with impaired spermatogenesis (Su *et al.*, 2008, Gould *et al.*, 2007) and ii) ERβ immunolocalization was found in sperm tails (Rago *et. al.,* 2006), further studies should be conducted to verify if estrogen can regulate sperm motility in all semen samples either normal or abnormal, as it does in normal sperm population (Aquila *et. al.,* 2004).

Regarding the ERβ *RsaI* (1082G→A) polymorphism distribution, no significant association was found with sperm concentration or sperm motility, probably due to the low frequency of this polymorphism. Oppositely, Aschim *et. al.* demonstrated that the frequency of heterozygous *Rsa*I AG genotype was three times higher in infertile men compared with the controls (men with sperm count . $5x10^6$ spermatozoa/ml). The heterozygous *Rsa*I AG genotype was associated with an approximately 20% reduction in LH concentration, compared with the wild-type *Rsa*I GG genotype in both controls and infertile men. However, in that study, the frequency of the *Rsa*I polymorphism was also very low and this frequency may also be an indicator of the adverse effects of this genotype on fertility.

In summary, although the small number of cases enrolled in this study may limit the power of our findings, is nevertheless indicative of the significance of the ERα 397T→C and 351A→G polymorphisms and the ERβ 1730A→G polymorphisms in semen quality. This study has shown, for the first time, association of both estrogen receptor genes with either motility or concentration, supporting that these genes might contribute to the efficiency of spermatogenesis. After the verification of our results in a larger population it could be possible the performance of molecular analysis of ERs' polymorphisms in human sperm and the appropriate genetic counselling to all couples undergoing assisted reproductive technologies when the male partner suffered from oligo- or asthenozoospermia.

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Table 1. Genotype frequencies of the study population. Correlation with sperm concentration and motility.
(normal sperm count men ≥20x10^6 spermatozoa/ml; oligozoospermic men <20x10^6 spermatozoa/ml, normal motility: grade A+B> 50%; asthenozoospermia grade A+B< 50%)

Polymorphism	**Genotype**	**Oligospermic Men**	**Normal Sperm Count Men**	**p-value**	**Sperm Motility**		**p-value**
					<50%	**>50%**	
XbaI	AA	17.2 %	15.3 %	ns	54.5 %	45.5 %	ns
	AG	44.8 %	50.6 %		60.3 %	39.7 %	
	GG	38 %	34.1 %		43.5 %	56.5 %	
PvuII	TT	20.7 %	23.5 %	ns	53.3 %	46.7 %	ns
	TC	48.3 %	47.1 %		59 %	41 %	
	CC	31 %	29.4 %		45 %	55 %	
AluI	AA	34.5 %	37.6 %	ns	54.2 %	45.8 %	ns
	AG	41.4 %	42.4 %		57.9 %	42.1 %	
	GG	24.1 %	20 %		42.3 %	57.7 %	
RsaI	GG	89.7 %	94.1 %	ns	52.5 %	47.5 %	ns
	GA	10.3 %	5.9 %		63.6 %	36.4 %	
	AA	0 %	0 %		0 %	0 %	

Table 2. Association of ERα and ERβ polymorphisms with sperm characteristics

Polymorphism	**Genotype**	**Sperm Motility**					
		<20x10^6 sperm/ml			**>20x10^6 sperm/m**		
		<50%	**>50%**	**p-value**	**<50%**	**>50%**	**p-value**
XbaI	AA	23.5 %	8.3 %	**0.045**	11.1 %	18.4 %	ns
	AG	50 %	25 %		58.3 %	44.9 %	
	GG	26.5 %	66.7 %		30.6 %	36.7 %	
PvuII	TT	26.5 %	8.3 %	**0.013**	19.4 %	26.5 %	ns
	TC	52.9 %	25 %		50 %	44.9 %	
	CC	20.6 %	66.7 %		30.6 %	28.6 %	
AluI	AA	32.4 %	41.7 %	Ns	41.7 %	34.7%	ns
	AG	47 %	41.7 %		47.2 %	38.8 %	
	GG	20.6 %	16.6 %		11.1 %	26.5 %	
RsaI	GG	82.4 %	100 %	Ns	97.2 %	91.8 %	ns
	GA	17.6 %	0 %		2.8 %	8.2 %	
	AA	0 %	0 %		0 %	0 %	

Figure Legends

Figure 1
Association of *XbaI* (351A→G) and ERα *PvuII* (397T→C) genotypes with sperm motility in men with sperm concentration $<20x10^6$ sperm/ml. ($p=0.013$ and $p=0.003$, respectively)

Figure 2
Association of ERα *PvuII* (397T→C) genotypes with sperm concentration in normal sperm count men ($p=0.016$).

Figure 3
Association of ERα *XbaI* (351A→G) genotypes with sperm concentration in normal sperm count men ($p=0.05$).

Figure 1

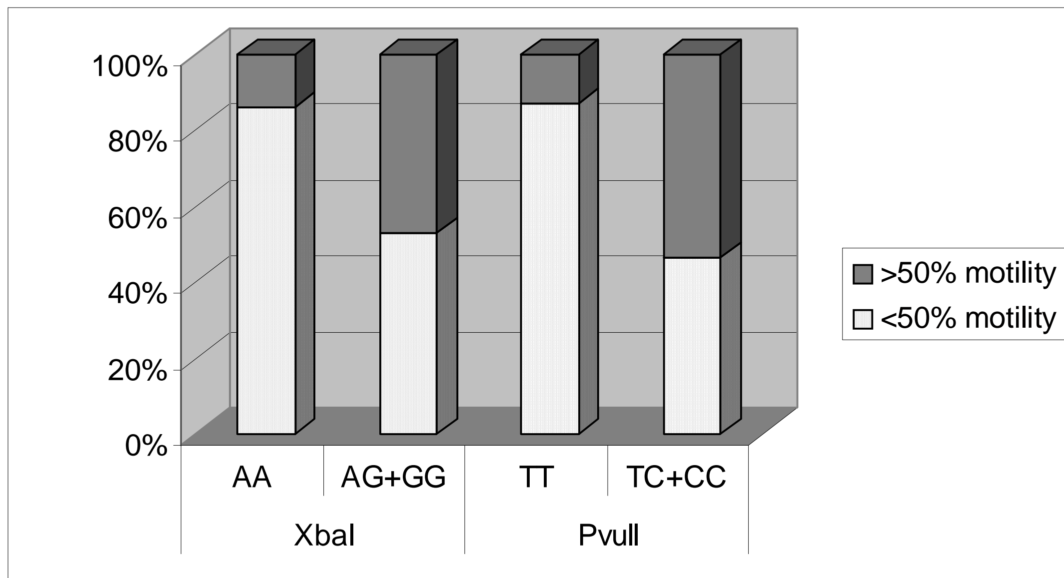

Figure 2

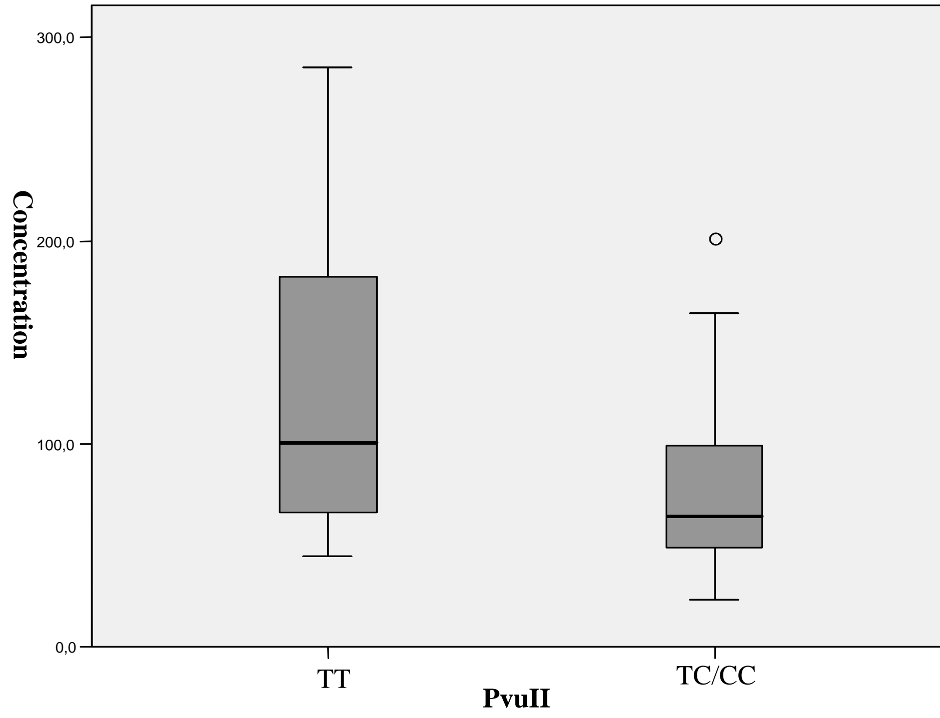

Figure 3