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ORIGINAL ARTICLE

The association of aromatase (*CYP19*) gene variants with sperm concentration and motility

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The irreversible transformation of androgens into oestrogens is catalysed by cytochrome P450 aromatase. In the present study, we explored the contribution of the (TTTA)$_n$ polymorphism in the aromatase gene (*CYP19*) to sperm concentration and motility. Ninety normozoospermic and 60 oligospermic men were examined during infertility examinations. DNA was extracted from spermatozoa, and the *CYP19* (TTTA)$_n$ polymorphism was genotyped by PCR. Genotype analysis revealed six *CYP19* (TTTA)$_n$ alleles with 7–12 repeats. The allelic distribution of the *CYP19* (TTTA)$_n$ polymorphism differed between normozoospermic and oligospermic men ($P<0.01$). Oligospermic men less frequently had long *CYP19* alleles than did normozoospermic men (25 and 37.8%, respectively; $P<0.02$). The higher frequency of short *CYP19* alleles in oligospermic men compared to normozoospermic men (43.3 and 28.3%, respectively; $P<0.01$) was primarily due to the distribution of the *CYP19* (TTTA)$_7$ allele. The *CYP19* (TTTA)$_7$ allele was associated with lower sperm concentration in normozoospermic men ($P<0.01$) and in the total study population ($P<0.01$); it was also associated with lower sperm motility in normozoospermic men ($P<0.05$) and in the total study population ($P<0.01$). In conclusion, the *CYP19* (TTTA)$_7$ allele probably impairs aromatase activity, which in turn alters aromatase and oestrogen levels in the testis, leading to decreased sperm concentration and motility. These findings support the significance of cytochrome P450 aromatase in human spermatogenesis and consequently in semen quality.
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Keywords: androgen; male infertility; oestrogen; semen quality; spermatogenesis

INTRODUCTION

The synthesis of steroid hormones and the production of spermatozoa are the main functions of the human testis. Steroid hormones affect normal testicular development and the preservation of spermatogenesis.[1] Their action is mediated by factors produced within the testis, among which aromatase is the key enzyme. Aromatase, which catalyses the conversion of androgens into oestrogens, is located in the endoplasmic reticulum of the brain, placenta, gonads and adipose tissue of humans.[2] Aromatase transcripts have been found in various testicular cells, including Leydig cells, Sertoli cells, spermatocytes and spermatids,[3] as well as in human ejaculated spermatozoa.[4,5]

The crucial role of aromatase in normal spermatogenesis and, consequently, in human reproduction, has been shown by male aromatase knockout (ArKO) mouse experiments. These ArKO mice lack a functional aromatase P450 enzyme and are thus unable to produce oestrogen from androgen. Although these mice were initially fertile without severe histological or morphological abnormalities of the testes, they presented a progressive, long-term deterioration of spermatogenesis.[6] In older ArKO mice, a progressive disruption of spermatogenesis was observed, resulting in a decrease in round and elongated spermatids.[7] Additionally, ArKO mice presented with increased apoptosis and reduced sperm maturation, resulting in the generation of spermatozoa with decreases in motility and the ability to fertilize oocytes.[8]

Cytochrome P450 aromatase is an enzymatic complex composed of two proteins, NADPH-cytochrome P450 reductase and cytochrome P450 aromatase. The human P450 aromatase is the product of a single gene, *CYP19* (cytochrome P450, family 19, subfamily A, polypeptide 1; PubMed GeneID: 1588) located on chromosome 15.[2] The *CYP19* gene spans more than 123 kilobases, with nine translated exons encoding a unique protein of 55 kDa and 11 untranslated exons in the beginning of the gene.[9] Recently, a total of 19 *CYP19* variations were identified, as follows: four in the coding exons, ten in the untranslated exons, six in the 5' untranslated region and one in the 3' untranslated region.[10]

The impact of *CYP19* gene variations on *CYP19* activity has been explored in a wide range of clinically important oestrogen-dependent disorders. The most studied polymorphism is a short tetranucleotide tandem repeat, (TTTA)$_n$, in intron 4 of the *CYP19* gene. This polymorphism has been implicated in steroid hormone regulation, leading to cardiovascular disease[11] and to enhanced vasomotor symptoms during the menopausal transition in women.[12] Short (TTTA)$_n$ alleles have been suggested to confer, *via* decreased aromatase activity, a genetic susceptibility to unexplained infertility and endometriosis,[13] to prenatal androgenisation leading to the development of polycystic ovary syndrome. phenotypes in adult life[14] and to abdominal obesity.[15] In contrast, long (TTTA)$_n$ alleles have been implicated in gynaecomastia.[16] In addition, many types of cancer, such as endometrial,[17]

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breast[18] and prostate,[19] have been associated with various *CYP19* $(TTTA)_n$ genotypes.

Cytochrome P450 aromatase contributes to normal sexual development and reproduction in humans by influencing oestrogen bioavailability and androgen maintenance. In addition, the $(TTTA)_n$ alleles of the *CYP19* gene affect aromatase activity. Keeping these functions of cytochrome P450 aromatase in mind, we investigated the effects of *CYP19* $(TTTA)_n$ polymorphisms on sperm concentration and motility.

MATERIALS AND METHODS

Subjects

The study population consisted of 90 normozoospermic and 60 oligospermic men who were referred to the IVF Unit of the Department of Obstetrics and Gynaecology, Medical School of Ioannina, Ioannina, Greece, for semen analysis. The stratification of men into the normozoospermic and oligospermic groups was based on World Health Organization (WHO, 1999) criteria.[20] Specifically, men with a sperm concentration $\geqslant 20\times10^6$ spermatozoa/ml, motility grade A+B>50 and >30% normal forms were characterized as normozoospermic.

Men suffering from hypogonadotropic hypogonadism, hydrocele, varicocele or obstructive syndromes of the seminal tract were excluded from the study, as were men with microdeletions of the long arm of the Y-chromosome or karyotype abnormalities and men taking spermatogenesis-impairing medications. Asthenozoospermic, oligoasthenoteratozoospermic and azoospermic men were also excluded from the study.

A questionnaire was administered to each subject to obtain information about his medical history, reproductive history, general lifestyle, job description, educational background and socioeconomic status. A physical examination was performed, and semen was analysed according to WHO guidelines.[20] Men were asked to abstain from sexual activity for 2–5 days prior to semen analysis. Two independent investigators performed blinded semen analysis. The average values of the two investigators were calculated, and in the event of inconsistency (over a 10% difference), a third assessment was performed.

In all patients, blood samples were drawn for the measurement of serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T) and oestradiol (E_2). Levels of total testosterone and serum LH, FSH and E_2 were determined by chemiluminescent microparticle immunoassay on an Abbott-ARCHITECT immunoanalyser (Abbott Laboratory, Abbott Park, IL, USA.). The coefficients of variation were 4% for total testosterone, 3.5% for LH, 4% for FSH and 6.5% for E_2.

The Ioannina University Hospital Ethics Committee approved the study protocol in accordance with the Declaration of Helsinki, and all participants gave informed consent.

DNA extraction

DNA was extracted from sperm according to a previously described protocol.[21] Briefly, approximately 7×10^6 spermatozoa were mixed with 1× phosphate-buffered saline and centrifuged at 600*g* for 6 min. In men with severe oligospermia, we used the highest number of spermatozoa that we were able to isolate for the DNA extraction. The supernatant was discarded and the precipitate was diluted in 200 μl 1× phosphate-buffered saline and centrifuged at 6700*g* for 1 s. Subsequently, 200 μl 1× phosphate-buffered saline, 15 μl 10% sodium dodecyl sulphate, 13 μl dithiothreitol and 0.2 mg proteinase K were added to the diluted precipitate, and the mix was incubated for 2 h. Then, 85 μl 6 mol l^{-1} NaCl was added, and the dilution was mixed and centrifuged at 9700*g* for 3.5 min. Finally, the supernatant was transferred to a clean tube, and 750 μl frozen absolute ethanol was added. The isolated DNA was preserved in 50 μl Tris-HCl EDTA buffer at 4 °C.

Genotype analysis

The *CYP19* $(TTTA)_n$ repeat region was amplified by the PCR with a forward primer (5′-GTTTGACTCCGTGAGTTTGA-3′) and a reverse primer (5′-CAACTCGACCCTTCTTTATG-3′). The thermal cycling included denaturation for 5 min at 94 °C, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 53 °C and 1.5 min at 72 °C, with a final extension of 10 min at 72 °C. The PCR products were separated by 10% polyacrylamide gel electrophoresis followed by silver staining, and the number of TTTA repeats in each allele was analysed by sequencing the appropriate PCR products.[22] Random sampling and sequencing were used for the assessment of quality control. All samples were run in duplicate along with positive and negative controls.

Statistical analysis

The statistical analysis of the differences in allele and genotype frequencies was performed using the chi-square test. The normal distribution of continuous parameters was analysed by the Kolmogorov–Smirnov test. Differences in continuous parameters were assessed by either a unpaired *t*-test for independent variables, the non-parametric Mann–Whitney U test or the Kruskal–Wallis test. A P value of <0.05 was considered statistically significant. All results are reported as the mean±standard deviation. All analyses used the SPSS statistical package (version 14.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Clinical characteristics of the study population

The clinical characteristics of the 90 normozoospermic and 60 oligospermic men are presented in Table 1. The sperm concentration and motility as well as the serum levels of FSH, LH and T differed between normozoospermic and oligospermic men ($P<0.001$) (Table 1).

Genotype analysis

The genotype analysis of the *CYP19* $(TTTA)_n$ polymorphism revealed six *CYP19* $(TTTA)_n$ alleles with 7–12 repeats. To analyse the effects of this polymorphism on sperm characteristics, the study population was divided into two subgroups using the $(TTTA)_9$ allele as a cutoff point:

Table 1 Characteristics of the study population

	Normozoospermic men	*Oligospermic patients*	P *value*
No. of patients	90	60	—
Age of patients (year)	35.9±6.8	34.8±7.6	ns
Body mass index (kg m^{-2})	27.4±5.1	26.8±5.4	ns
Smoking (%)	36	33	ns
Sperm concentration ($\times10^6$ spermatozoa/ml)	79.6±55.2	8.3±5.4	<0.001
Sperm motility (%)	64.7±9.3	36.4±18.5	<0.001
FSH (mIU ml^{-1})	5.1±1.4	9.8±1.9	<0.001
LH (mIU ml^{-1})	4.2±1.2	11.0±1.8	<0.001
T (ng dl^{-1})	758±31	425±27	<0.001
E_2 (pg ml^{-1})	42.0±6.1	39.0±7.8	ns

Abbreviations: E_2, oestradioll; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ns, not significant; T, testosterone.
Normozoospermic men: sperm concentration $\geqslant 20\times10^6$ spermatozoa/ml; normal motility >50%. Data are presented as mean±standard deviation.

Table 2 The allelic distribution of the *CYP19* (TTTA)$_n$ polymorphism in normozoospermic and oligospermic men

CYP19 *(TTTA)*$_n$ *allele*	*Normozoospermic men,* n *(%)*	*Oligospermic men,* n *(%)*	P *value*
7	51 (28.3)	52 (43.3)	<0.01
8	43 (23.9)	20 (16.7)	
9	18 (10)	18 (15)	
10	29 (16.1)	10 (8.3)	
11	30 (16.7)	18 (15)	
12	9 (5)	2 (1.7)	

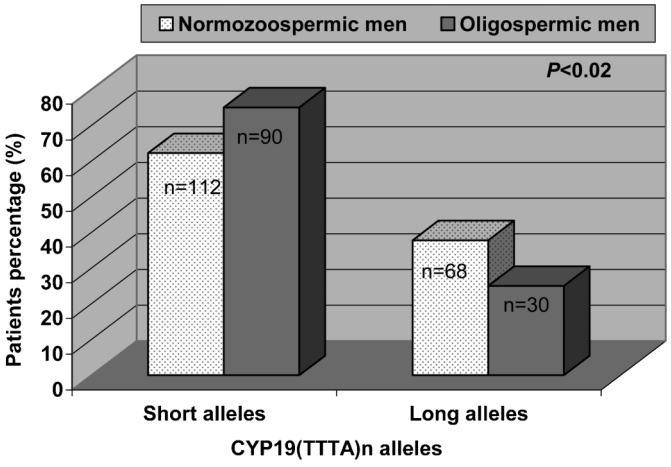

Figure 1 The distribution of short and long *CYP19* (TTTA)$_n$ alleles in normozoospermic and oligospermic men using chi-square analysis ($P<0.02$) (short alleles: alleles with $\leqslant$9 TTTA repeats; long alleles: alleles with >9 TTTA repeats).

men with short *CYP19* genotypes (homozygous for $\leqslant$9 TTTA repeats) and men with long *CYP19* genotypes (homozygous for >9 TTTA repeats). The same cutoff allele, which is near the median repeat length of normozoospermic (8.7±1.1) and oligospermic men (8.4±1.2), has been used in previous studies exploring the distribution of the *CYP19* (TTTA)$_n$ polymorphism.[14,22]

The *CYP19* (TTTA)$_n$ allelic distribution differed between normozoospermic and oligospermic men ($P<0.01$) (Table 2). Oligospermic men (sperm concentration $<20\times10^6$ spermatozoa/ml) presented long *CYP19* (TTTA)$_n$ alleles in their genotype less frequently than did normozoospermic men (25% versus 37.8%, respectively; $P<0.02$) (Figure 1). When we separately focused on the distribution of each *CYP19* allele, the high frequency of short *CYP19* alleles in oligospermic men was mainly due to the *CYP19* (TTTA)$_7$ allele distribution (65.2% versus 46.7%, in oligospermic and in normozoospermic men, respectively; $P<0.01$) (Figure 2).

To exclude any mosaicism or variation of the *CYP19* alleles in sperm, we randomly extracted DNA from peripheral blood samples of half the study population and analysed the *CYP19* (TTTA)$_n$ polymorphism. No difference in genotype was found when we compared the results of the matched blood and sperm DNA samples.

We separately analysed each *CYP19* genotype and allele, in both normozoospermic and oligospermic men. There were no associations found between these genotypes and alleles and the serum levels of FSH, LH, T and E_2, and there were no associations with smoking and body mass index.

The association of *CYP19* (TTTA)$_n$ polymorphisms with sperm concentration

When *CYP19* (TTTA)$_n$ alleles were correlated with sperm concentration, a significant association was found between the (TTTA)$_7$ allele and low sperm concentration in normozoospermic men. Men with no *CYP19* (TTTA)$_7$ allele in their genotype had a mean sperm concentration of $92.2\pm34.5\times10^6$ spermatozoa/ml, whereas those with the *CYP19* (TTTA)$_7$ allele had lower sperm concentrations ($45.8\pm24.5\times10^6$ spermatozoa/ml, odds ratio=2.65, 95% confidence interval=1.37–4.85, $P<0.01$) (Figure 3).

The same association was confirmed in the total study population. Men homozygous and heterozygous for the *CYP19* (TTTA)$_7$ allele had lower sperm concentrations ($26.7\times10^6\pm23.4\times10^6$ and $46.2\times10^6\pm41.2\times10^6$ spermatozoa/ml, respectively) than men with no *CYP19* (TTTA)$_7$ allele ($69.3\pm54.9\times10^6$ spermatozoa/ml) ($P<0.01$). In contrast, no association was found between this allele and sperm concentration in oligospermic men.

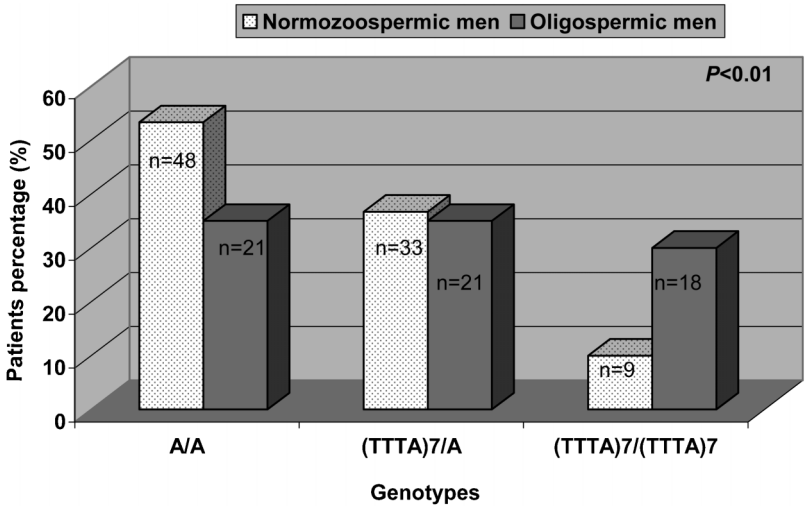

Figure 2 The distribution of the *CYP19* (TTTA)$_7$ allele in the genotypes of normozoospermic and oligospermic men using chi-square analysis ($P<0.01$) (A: alleles with $\geqslant$7 TTTA repeats).

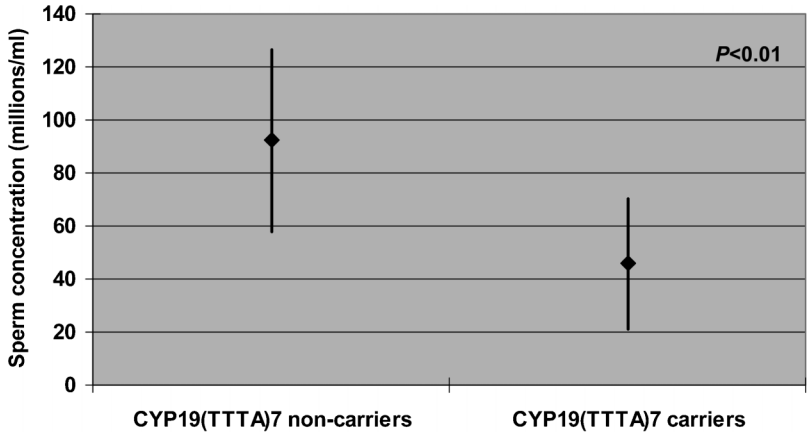

Figure 3 The association of the *CYP19* $(TTTA)_7$ allele with sperm concentration in normozoospermic men ($P<0.01$).

The association of the *CYP19* $(TTTA)_n$ polymorphism with sperm motility

When *CYP19* $(TTTA)_n$ alleles were correlated with sperm motility, a significant association was found between the *CYP19* $(TTTA)_7$ allele and low sperm motility in normozoospermic men. Men with no *CYP19* $(TTTA)_7$ allele had higher sperm motility (69.2±4.5%) than those with the *CYP19* $(TTTA)_7$ allele (63.3%±6.7% motility in heterozygotes and 60.5%±4.9% in homozygotes) ($P<0.05$) (Figure 4).

The same association was confirmed in the total study population. Men with the *CYP19* $(TTTA)_7$ allele had lower sperm motility (43.2±18.6% in heterozygotes and 38.3±19.8% in homozygotes) than men with no *CYP19* $(TTTA)_7$ allele in their genotype (54.5±16.7%) ($P<0.01$). No association was found between this allele and sperm motility in oligospermic men.

DISCUSSION

The crucial roles of steroid hormones in male reproduction have been studied extensively over the last several decades. The effects of oestrogen on spermatogenesis and spermiogenesis are significant, involving the testicular oestrogen receptors (ERs), which modulate the transcription of specific genes. Oestrogen production and androgen bioavailability are regulated by cytochrome P450 aromatase activity. Although the role of the P450 aromatase in human spermatogenesis is still under investigation, the noteworthy decrease in round and elongated spermatids after the administration of an aromatase inhibitor in rat[23] and monkey,[24] and the P450 aromatase localisation in the germinal epithelium of the adult mouse testis, primarily in the Golgi region of round spermatids, throughout the cytoplasm of elongating spermatids and along the flagella of late spermatids,[25] support the idea that P450 aromatase is important for spermatogenesis in humans'.

The current study examined the allelic distribution of the *CYP19* $(TTTA)_n$ polymorphism in 150 men, 60 oligospermic and 90 normozoospermic. Our data indicate that the $(TTTA)_n$ polymorphism of the *CYP19* gene may influence human spermatogenesis and, consequently, sperm quality. The allelic distribution of this polymorphism was correlated with important differences between normozoospermic and oligospermic men. In particular, the *CYP19* $(TTTA)_7$ allele was associated with lower sperm concentration and motility in normozoospermic men and in the total study population.

How might the *CYP19* $(TTTA)_n$ polymorphism influence sperm concentration? The transformation of androgens to oestrogens is catalysed by aromatase. Consequently, the level of oestrogen and androgen regulation is based on aromatase efficiency. In this study, oligospermic men were found to more frequently have short *CYP19* $(TTTA)_n$ alleles compared to normozoospermic men. In addition, an association between reduced sperm concentration and the *CYP19* $(TTTA)_7$ allele, both in normozoospermic men and in the total study population, was observed. This association supports the hypothesis

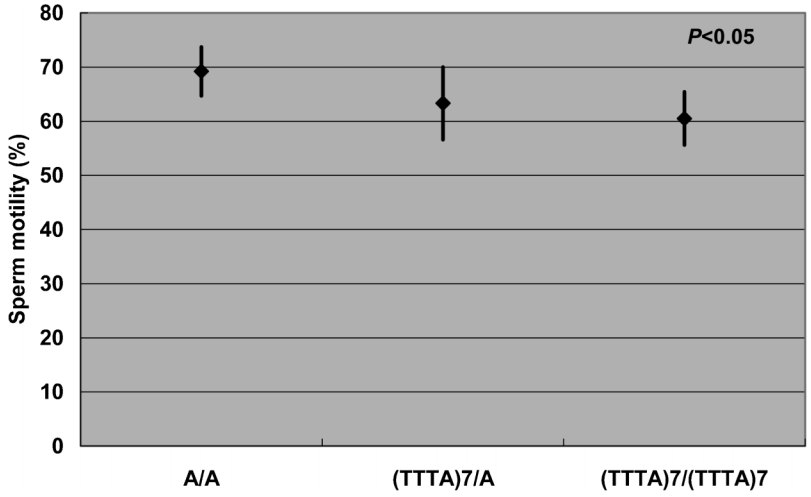

Figure 4 The association of the *CYP19* $(TTTA)_7$ allele with sperm motility in normozoospermic men ($P<0.05$) (A: *CYP19* $(TTTA)_n$ alleles with >7 TTTA repeats).

that short *CYP19* $(TTTA)_n$ alleles, and especially the *CYP19* $(TTTA)_7$ allele, may influence the transcription, mRNA stabilisation or post-translational expression regulation of aromatase, causing an alteration in oestrogen levels that might lead to altered hormone levels in the testis and impaired spermatogenesis. The association of *CYP19* $(TTTA)_7$ allele with altered hormone levels in the testis has been also suggested in previous studies, demonstrating that this allele is associated with lower concentrations of testicular oestrone, oestrone sulphate and oestradiol as a result of reduced aromatase activity.[26,27]

In vitro studies have provided evidence of an association between long *CYP19* alleles and enhanced aromatase activity.[28,29] Berstein *et al.*[28] reported increased oestrogen biosynthesis due to high aromatase activity in endometrial tumours from $(TTTA)_{11}/(TTTA)_{11}$ and $(TTTA)_{11}/(TTTA)_{12}$ genotype carriers,[28] and Stratakis *et al.*[29] reported the presence of the *CYP19* $(TTTA)_{11}$ allele in all members of a family who had symptoms of aromatase excess syndrome. Considering both the above observations and the findings of the current study, we propose that short *CYP19* alleles are associated with decreased aromatase activity and altered testicular oestrogen levels, which are associated with reduced numbers of spermatogonia, spermatocytes and spermatids. In contrast, our findings revealed no association between *CYP19* alleles and serum oestrogen levels, supporting the hypothesis that serum oestrogen levels do not have any direct effect on spermatogenesis.

The *CYP19* $(TTTA)_n$ polymorphism may also influence sperm motility, apart from its effect on sperm concentration. Men heterozygous or homozygous for the *CYP19* $(TTTA)_7$ allele were found to have lower sperm motility compared to those without a *CYP19* $(TTTA)_7$ allele in their genotype. This finding could be explained by the decrease in aromatase activity associated with short *CYP19* alleles.[28,29] Indeed, previous studies have suggested a role for aromatase in sperm motility. Men with an aromatase deficiency[30] and aromatase knockout mice[31] have been reported to have decreased sperm motility, whereas aromatase activity has been found to be 50% greater in motile compared to immotile spermatozoa.[32] In addition, the amount of P450 aromatase transcripts was found to be 30% lower in immotile versus motile spermatozoa of healthy men,[33] and a two-fold decrease in aromatase transcripts was observed in spermatozoa from a group of infertile men.[34] Recently, Galeraud-Denis *et al.*[35] reported that the spermatozoa of asthenospermic, teratospermic and asthenoteratospermic patients have 44, 52 and 67% decreases, respectively, in the amount of aromatase transcripts as compared to controls.

However, sperm motility is a complicated process and is not based solely on aromatase activity. Oestrogens that are produced in the testis have been found to stimulate sperm motility in a paracrine or autocrine fashion, regulating the reabsorption of luminal fluid in the head of the epididymis. Disruption of this essential function causes sperm to enter the epididymis diluted, rather than concentrated, resulting in infertility.[36] Reduced sperm motility is thought to be the result of impaired fluid reabsorption within the efferent ducts of the testis,[37] altering pH and capacitation. By influencing aromatase activity, the *CYP19* $(TTTA)_n$ polymorphism probably alters testicular oestrogen levels and, consequently, the reabsorption of luminal fluid in the epididymis. Therefore, we suggest that the *CYP19* $(TTTA)_7$ allele, which is associated with low aromatase activity, causes an imbalance in testicular oestrogen levels leading to impaired reabsorption of luminal fluid in the head of the epididymis and decreased sperm motility.

It is noteworthy that two immunoreactive sites for aromatase have been found in ejaculated spermatozoa; one colocalizes with tubulin to the tail and with a specific marker of the midpiece (CD 46), and the other localizes to the highly fluorescent zone of the head,[35] suggesting a role for aromatase in the regulation of sperm motility. The coexistence of aromatase, ERs and mitochondria in the midpiece of spermatozoa[38,39] explains why locally produced oestrogens may influence sperm motility. Recent data have indicated a mechanism by which oestrogens can increase mitochondrial activity *via* nuclear respiratory factor-1 transcription in human ejaculated spermatozoa, driving the spermatozoa to higher motility.[40] The mediation of oestradiol's effects on spermatozoa by both genomic and non-genomic activation[41] could explain why membrane-associated ERs mediate the stimulatory action of oestrogens on spermatozoa.[42] A membrane-associated ER with specificity for oestradiol is mainly concentrated in the central part of the tail,[43] supporting the role of ERs in sperm motility regulation. The reduced aromatase activity of *CYP19* $(TTTA)_7$ allele carriers probably influences the testicular oestrogen levels, which in turn may decrease mitochondrial activity in spermatozoa, leading to low sperm motility.

In conclusion, the *CYP19* $(TTTA)_n$ polymorphism influences aromatase activity and, consequently, aromatase and oestrogen levels in the testis. Spermatogenesis and sperm motility regulation are complicated processes in which the activity of aromatase and ERs cooperate, influencing testicular oestrogen levels.[44] Although the small number of subjects enrolled in this study may limit the impact of our findings, our results are nevertheless indicative of the significance of the *CYP19* $(TTTA)_n$ polymorphism in semen quality. This study has shown, for the first time, an association between a cytochrome P450 aromatase gene polymorphism with sperm concentration and motility. The validation of our results in larger patient groups could provide evidence for the influence of aromatase on the on the fundamental characteristics of sperm.

AUTHOR CONTRIBUTIONS

L. Lazaros is the main investigator and article writer, responsible for conception of the study, N. Xita contributed to acquisition, analysis and interpretation of data, A. Kaponis contributed to study design, sample collection and revision of the article, E. Hatzi is an investigator, carried out semen analysis and contributed to study execution, N. Plachouras contributed to sample collection and revision of the article, N. Sofikitis contributed to study design and revision of the article, K. Zikopoulos contributed to sample collection and revision of the article, and I. Georgiou is the group leader, responsible for conception and design of the study, semen analysis and revision of the article.

COMPETING FINANCIAL INTERESTS

Authors declare no financial interests.

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