Aromatase gene (*CYP19A1*) variants, female infertility and ovarian stimulation outcome: a preliminary report

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Abstract

Progress has been made towards ascertaining the genetic predictors of ovarian stimulation in IVF. Aromatase cytochrome P450, encoded by the *CYP19A1* gene, catalyses a key step in ovarian oestrogen biosynthesis. Hence, the aromatase gene is an attractive candidate for genetic studies. This study aimed to examine the genetic influences of *CYP19A1* TCT trinucleotide insertion/deletion (Ins/Del) and (TTTA)n microsatellite intronic polymorphisms on ovarian stimulation outcome and aetiology of female infertility. IVF patients (n = 152) underwent ovarian stimulation according to recombinant FSH and gonadotrophin-releasing hormone antagonist protocol. Del/Del homozygous patients with shorter TTTA repeats exhibited decreased ovarian FSH sensitivity in ovarian stimulation, which may reflect variations in aromatase gene expression during early antral follicle development. Accordingly, this study demonstrates correlations between Del allele and shorter (TTTA)n repeat sizes with smaller ovaries (r = −0.70, P = 0.047) and fewer antral follicles (r = 0.21, P = 0.018) on days 3–5 of spontaneous menstrual cycle, respectively. Furthermore, Del variation linked with low-repeat-number (TTTA)n alleles are involved in enhanced genetic susceptibility to unexplained infertility (adjusted OR = 4.33, P = 0.039) and endometriosis (r = −0.88, P = 0.026), which corroborates evidence on the overlapping patient profiles of ovarian dysfunction in both types of female infertility.

Keywords: aromatase, female infertility, IVF, ovarian stimulation

Introduction

Oestrogens and FSH act synergistically to induce follicular growth and maturation. Oestrogen biosynthesis depends on the collaboration between follicular theca and granulosa cells. Androgens produced in steroidogenic theca cells diffuse into the granulosa layer and are then aromatized into oestrogens (Ryan and Petro, 1966). The conversion of androgens to oestrogens is catalysed by FSH-inducible aromatase cytochrome P450 (Whitlock, 1986). Ovarian aromatase activity is continuously required for follicular cycle progression from growth and maturation to ovulation, and even for luteal function (Ryan, 1982).

IVF includes administration of FSH to stimulate multiple follicle development by suppressing the dominant follicle selection and the atresia of subordinate antral follicles. Age and reduced ovarian reserve negatively impact the ovarian response to FSH stimulation during ovarian stimulation in IVF (Kligman and Rosenwaks, 2001). Previous work demonstrated a causative association between anti-FSH autoantibodies and poor ovarian stimulation outcome (Haller *et al.*, 2008). Additionally, variations in FSH receptor (*FSHR*) and oestrogen receptor (*ESR1*) genes influence FSH activity during ovarian stimulation (Georgiou *et al.*, 1997; Perez Mayorga *et al.*, 2000; Altmäe *et al.*, 2007). Another focus of interest is the aromatase enzyme, because it catalyses the key step in ovarian oestrogen biosynthesis. Thus, aromatase is an attractive candidate for genetic studies.

Aromatase is encoded by the *CYP19A1* gene (15q21.1), spanning over 123 kb and comprised of nine (II-X) coding exons. Aromatase is expressed in ovarian, placental, testicular, adipose, bone and brain tissues (Sebastian and Bulun, 2001). Tissue specificity is regulated by the use of nine alternate untranslated first exons located in the large 93 kb gene regulatory unit. Ovarian aromatase expression is controlled by promoter PII within 1 kb upstream of exon II (Sebastian and Bulun, 2001).

Inappropriate activation of promoters may underlie the aetiology of oestrogen-driven diseases, such as endometriosis and breast cancer. Although eutopic endometrial tissue lacks aromatase expression, elevated *CYP19A1* transcription via the recruitment of ovarian-specific promoter PII is characteristic of pelvic endometriotic lesions (Noble *et al.*, 1996; Zeitoun *et al.*, 1999). In addition to dysregulated promoter activation, several *CYP19A1* gene variants increase susceptibility to certain diseases. Common $(TTTA)_n$ polymorphism comprised of 7–13 repeats in intron 4 has attracted the most attention. Polycystic ovarian syndrome (PCOS) is described by an accumulation of incompletely developed follicles due to low concentrations of local oestrogens and aromatase enzymatic activity. Women with PCOS possess, at greater frequency, shorter *CYP19A1* alleles with ≤9 TTTA repeats. Importantly, these PCOS patients show the highest serum testosterone and testosterone/oestradiol ratio during the early follicular phase of the menstrual cycle (Xita *et al.*, 2008). In contrast, longer alleles of 10 or 12 repeats have been suggested as breast cancer risk alleles with excessive aromatase activity (Kristensen *et al.*, 1998; Haiman *et al.*, 2000).

TCT trinucleotide insertion (Ins) or deletion (Del) variation occurs upstream of $(TTTA)_n$ microsatellite. The three base pair deletion segregates exclusively with $(TTTA)_7$ variant, and generates two alleles: Del-$(TTTA)_7$ and Ins-$(TTTA)_n$ (Probst- Hensch *et al.*, 1999). Del-$(TTTA)_7$ associates with increased follicular phase serum testosterone and testosterone/oestradiol ratio in premenopausal women, suggesting lower ovarian aromatase activity (Baghaei *et al.*, 2003).

While the prevalent interest in *CYP19A1* gene variants has emphasized associations with cancers of female reproductive organs, other possible outcomes also seem obvious targets to study. The described genetic variations in *CYP19A1* may affect gene expression or aromatase enzymatic activity, and thus result in alterations in regulation of folliculogenesis that impact

ovarian stimulation outcome during infertility treatment. Identification of the genetic predictors of ovarian response in IVF would enable clinicians to individualize ovarian stimulation regimen, minimize the risks of cycle cancellation and ovarian hyperstimulation, and maximize the chance of pregnancy. The present study examines the associations between *CYP19A1* $(TTTA)_n$ repeat and Ins/Del polymorphisms, and ovarian stimulation outcome among Estonian IVF patients.

Materials and methods

Patients

The study was approved by the Ethics Committee of the University of Tartu, and informed consent was obtained from all 152 participating normally ovulating women undergoing IVF treatment. The patients were 34.0 ± 4.9 (mean ± SD) years old, and had been infertile for at least a year prior to entering the study. Their indications for IVF were as follows: tubal factor infertility (44.1%, $n = 67$), male factor infertility (31.6%, $n = 48$), endometriosis (9.2%, $n = 14$), unexplained infertility (9.2%, $n = 14$), and infertility due to other reasons such as uterine myomas (5.9%, $n = 9$). The endometriosis stages according to the American Society for Reproductive Medicine revised classification system (ASRM, 1997) were as follows: minimal to mild (III) stages in 10 patients and moderate to severe (IIIIV) stages in four patients. *CYP19A1* $(TTTA)_n$ allelic variants are known to interfere with follicular steroidogenic properties in the genesis of polycystic ovarian phenotype (Xita *et al.*, 2008), and thus PCOS patients were excluded from the study.

Mean ultrasound parameters for right and left ovaries (volume and early antral follicle count) and serum FSH concentration (9.3 ± 5.3 IU/l) were determined between days 3–5 of

spontaneous menstrual cycle, which allowed indirect ovarian follicular reserve assessment. Ovarian volume (4.9 ± 2.1 cm^3) was calculated using the formula: 0.5(A B C), where A is the longitudinal, B the anteroposterior and C the transverse diameter of the ovary (Sample *et al.*, 1977). Early antral follicles (4.5 ± 1.4 follicles) were counted in longitudinal cross-section. All hormonal analyses were conducted using chemiluminescence immunoassay (Immulite 2000; Siemens Healthcare Diagnostics, Deerfield, IL, USA). The within-run (intra-assay) precision coefficients of variation (CV) ranged from 2.3 to 3.7 and 6.3 to 15.0% and the total *(*inter-assay*)* precision CV ranged from 5.4 to 6.7 and 6.4 to 16.0% for FSH and oestradiol respectively. Associations between ovarian reserve parameters and ovarian stimulation variables have also been described in previous studies (Altmäe *et al.*, 2007; Haller *et al.*, 2008).

Ovarian stimulation regimen and IVF

Ovarian stimulation was conducted according to the gonadotrophin-releasing hormone (GnRH) antagonist regimen. All patients commenced ovarian stimulation with the recombinant FSH (Gonal-F; Serono, Rome, Italy) mean starting dose of 178.3 ± 40.6 IU on day 1–3 of menses, continuing 9.6 ± 0.7 days until 1 day before human chorionic gonadotrophin (HCG) (Ovitrelle; Serono) administration. Daily GnRH antagonist administration (0.25 mg, Cetrotide; Serono or Orgalutran; N.V. Organon, Oss, The Netherlands) was initiated when at least one follicle reached the size of 14 mm. The GnRH antagonists were given for up to 4–5 days, including the day of HCG administration. Final follicular maturation was achieved using 250 g of HCG, followed by ovarian puncture 36 h later.

The number of follicles punctured at oocyte retrieval (14.5 ± 6.6) and the number of cumulusoocyte complexes obtained (12.4 ± 6.5) were counted for all participants. Serum oestradiol concentrations on the day of oocyte retrieval (4159.8 ± 4620.3 pmol/l), serum oestradiol concentration per punctured follicle at oocyte retrieval (295.1 ± 264.6 pmol/l) and serum oestradiol concentration per oocyte retrieved (388.9 ± 538.4 pmol/l) were also determined. In addition, follicular fluid oestradiol concentration (2375.4 ± 6924.1 nmol/l) was determined for all women.

Both IVF (45.4%, *n* = 69) and intracytoplasmic sperm injection (ICSI, 54.6%, *n* = 83) patients participated. The number of mature oocytes (10.1 ± 5.6) was calculated for both IVF and ICSI patients. The maturity of IVF oocytes was assessed 1 day after insemination by counting the fertilized and unfertilized metaphase II (M II) oocytes. ICSI oocytes were considered mature if they had reached M II stage by 4–6 h after oocyte retrieval. The total number of embryos with two pronuclei (embryos = 7.1 ± 4.1) was calculated 16–18 h after microinjection or insemination. The patients had, on average, 3.0 ± 2.8 (42.3 ± 29.6%) good-quality day 2 embryos, characterized by having at least four blastomeres and <20% cellular fragments.

The following parameters were calculated from the total amount of FSH used for ovarian stimulation (1893.5 ± 482.5 IU) to determine the amount of FSH (IU) administered: (i) per day (196.0 ± 40.8 IU); (ii) to mature one ovarian puncture follicle (184.6 ± 158.1 IU); (iii) to obtain one oocyte (239.2 ± 228.4 IU); (iv) per mature oocyte (303.3 ± 308.8 IU), (v) per embryo (393.3 ± 355.3 IU); and (vi) per good-quality embryo (835.7 ± 693.8 IU).

Two day-2 embryos were transferred into the uterus in the majority (87.6%) of IVF and ICSI cycles (2.1 ± 0.3 embryos per transfer). Vaginal progesterone (Lugesteron; Leiras, Turku, Finland) was used for luteal support. Single (*n* = 31) and twin (*n* = 16) clinical pregnancies

were recognized by the presence of gestational sac(s) with fetal heartbeat on transvaginal sonography at 6–7 weeks of gestation. Implantation (19.6%) and clinical pregnancy (30.9%) rates were calculated per embryo transfer.

CYP19A1 Ins/Del and $(TTTA)_n$ genotyping

Genomic DNA was extracted from peripheral EDTA blood using the salting-out method (Aljanabi and Martinez,1997). Polymerase chain reaction (PCR) of *CYP19A1* region encompassing both TCT Ins/Del and $(TTTA)_n$ polymorphisms was accomplished with fluorescently labelled forward (5-JOE-GGTAAGCAGGTACTTAGTTAG-3) and reverse (5-CAAGGTCGTGAGCCAAGGTC-3) primers. Amplification of 50 ng DNA was performed in a total volume of 15 l containing 0.25 mol/l dNTPs (MBI Fermentas, Vilnius, Lithuania), 2.5 mmol/l MgCl , 1 PCR buffer (Solis BioDyne, Tartu, Estonia), 10 pmol of primers (Metabion, Martinsried, 0 Germany) and 1U HotStart thermostable DNA polymerase HotFirePol (Solis BioDyne), in an Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The reactions were initiated with DNA denaturation and enzyme activation at 96C (10 min), followed by 35 cycles of denaturation at 96C (30 s), annealing at 57C (30 s), elongation at 72C (30 s), and final extension at 72C (5 min). The sizes of fluorescently labelled PCR products were estimated using ABI Prism 377 automated DNA sequencer and Genescan 2.1 software (PE Applied Biosystems, Forster City, CA, USA). Rox 500 (PE Applied Biosystems) was used as an internal size standard. DNA sequencing was used to verify the results of fragment size analysis in 8.0% of patients, using forward (5-TCATTACAGCTCTCGATTCG-3) and reverse (5-CAAGGTCGTGAGCCAAGGTC-3) primers.

Statistical analysis

Linear parameters are reported as mean ± SD. R2.3.1A Language and Environment software (Free Software Foundation, Boston, MA, USA) was used for linear and logistic regression analyses. Regression coefficients derived from linear regression analyses are reported in the table and text as *r*-values. Biallelic mean of $(TTTA)_n$ repeat was used in statistical analyses, representing the arithmetic mean of two parental *CYP19A1* variants. Women with tubal factor infertility were used as the control group. PS program (PS Power and Sample Size Calculations, Free- Software, Version 2.1.30, 2003, Nashville, TN, USA) was used for statistical power calculations. Statistical significance was set at $P < 0.05$ for all tests.

Results

CYP19A1 allelic variants and aetiology of female infertility

The distribution of *CYP19A1* $(TTTA)_n$ and Ins/Del allele frequencies is shown in **Figure 1**. $(TTTA)_n$ microsatellites ranged from 7 to 13 repeats and Del segregated only with $(TTTA)_7$. The two most prevalent allelic variants were $(TTTA)_{11}$ (34.9%) and Del-$(TTTA)_7$ (33.2%). Del and Ins alleles occurred with incidences of 33.2 and 66.8% respectively, while genotypes were distributed as follows: Ins/Ins (43.4%, *n* = 66), Ins/Del (46.7%, *n* = 71) and Del/Del (9.9%, *n* = 15). The most common combined $(TTTA)_n$ and Ins/Del genotypes were Del-$(TTTA)_7$/ $(TTTA)_{11}$ (23.7%) and $(TTTA)_{11}$/$(TTTA)_{11}$ (11.8%).

The average biallelic mean of $(TTTA)_n$ variation was 8.9 ± 1.3 repeats. Linear regression models were used to examine the associations between $(TTTA)_n$ biallelic means and the

causes of female infertility. Patients with endometriosis showed markedly shorter biallelic means of $(TTTA)_n$ repeats (8.3 1.1 repeats, $r = 0.88$, $P = 0.026$) when compared with the control group of women with tubal factor infertility (9.1 1.4 repeats). Female patients with unexplained and male factor infertility showed $(TTTA)_n$ length means similar to the control group.

The possible role of *CYP19A1* Ins/Del variation in the aetiology of female infertility was studied by applying logistic regression models. The presence of Del allele appeared as a genetic risk factor for unexplained infertility (Del/Del and Ins/Del frequency of 78.6%, odds ratio (OR) = 3.78, $P = 0.056$) when compared with the tubal factor infertility group (Del/Del and Ins/Del frequency of 49.3%). Del allele showed a significant relationship (OR = 4.33, $P = 0.039$) with unexplained infertility when the model was further corrected for early-follicular-phase serum FSH concentrations. Other causes of infertility were, however, unrelated to Ins/Del variant.

CYP19A1 variants and ovarian stimulationIVF outcome

CYP19A1 $(TTTA)_n$ biallelic means showed a positive correlation ($r = 0.21$, $P = 0.018$) with follicular count at days 3–5 of spontaneous menstrual cycle irrespective of patient's age. The ovarian volume and serum FSH of early follicular phase, also recommended as ovarian reserve markers, were unrelated to *CYP19A1* $(TTTA)_n$ polymorphic locus. During ovarian stimulation, age-adjusted linear regression model revealed a negative correlation between *CYP19A1* $(TTTA)_n$ biallelic means and the amounts of FSH used to mature one ovarian puncture follicle ($r = 18.38$, $P = 0.039$). The $(TTTA)_n$ biallelic means were 9.0 ± 1.3 and 8.8 ± 1.3 in the groups of women with and without clinical pregnancy respectively. Age adjusted

logistic regression model was not powerful enough (<80.0%) to suggest a significant association between 0.2 repeats of difference in $(TTTA)_n$ biallelic mean and increased chance of pregnancy (OR = 1.07, not significant).

Correlations between Ins/Del variation and clinical parameters influencing the outcome of ovarian stimulation were assessed with linear regression models that accounted for patient's age. Women with at least one Del allele (Del/Del and Ins/ Del genotypes) possessed markedly smaller ovaries (4.6 1.8 cm^3, $r = 0.70$, $P = 0.047$) compared with women with Ins/Ins genotype (5.3 2.4 cm^3). On the contrary, Ins/Del variation did neither predict serum FSH concentration nor follicle count at days 3–5 of the spontaneous menstrual cycle.

Associations between Ins/Del genotypes and ovarian stimulation variables are presented in **Table 1**. Women with Ins/Del and Ins/ Ins genotypes needed lower FSH doses to mature one ovarian puncture follicle (Ins/Del, $r = 89.67$, $P = 0.024$ and Ins/Ins, $r = 101.27$, $P = 0.011$) and to obtain one mature oocyte (Ins/Del, $r = 240.84$, $P = 0.004$ and Ins/Ins, $r = 211.61$, $P = 0.012$) when compared with patients with the reference Del/Del genotype according to age-adjusted linear regression models. In addition, Ins/Del heterozygotes tended to yield more oocytes ($r = 3.04$, $P = 090$) and mature oocytes ($r = 2.58$, $P = 0.094$), while carriers of both Ins/Del and Ins/Ins genotypes required, albeit not significantly, lower doses of FSH to obtain one oocyte (Ins/Del, $r = 104.38$, $P = 0.084$, and Ins/Ins, $r = 105.03$, $P = 0.083$) if contrasted with the reference Del/Del patients. Contrary to the expectation, IVF patients with Ins/Ins genotype showed marginally lower serum oestradiol ($r = 2580.60$, $P = 0.062$) and substantially reduced serum oestradiol per follicle punctured ($r = 173.90$, $P = 0.032$) as shown by linear regression models adjusted by ovarian volume. Age adjusted logistic regression models did not demonstrate any relation between Ins/Del gene variants and IVF pregnancy outcome: clinical pregnancy rates for patients with Ins/Ins 27.0%, Ins/Del 33.8%

and Del/Del 40.0% genotypes were observed. However, these statistical models were insufficiently powered (<80.0%) to conclusively rule out the lack of association between *CYP19A1* Ins/Del and IVF pregnancy success.

Discussion

Progress has been made towards ascertaining the genetic predictors of ovarian stimulation success. The genes involved in steroid biosynthesis and hypothalamicpituitaryovarian axis that possess numerous functional polymorphisms are promising targets for genetic studies. Along with substantial impacts on ovarian stimulation variables, these variations also play crucial roles in the pathogenesis of certain forms of female infertility. The present study is the first one to show associations between *CYP19A1* gene variants and the outcome of ovarian stimulation- IVF in normally ovulating infertile women. Patients with shorter *CYP19A1* $(TTTA)_n$ repeats and Del/Del homozygosity exhibit decreased ovarian FSH sensitivity during ovarian stimulation, along with the greater risk for endometriosis and unexplained infertility respectively.

The functional importance of linked intronic $(TTTA)_n$ and Ins/Del genetic markers is far from completely understood. Gene introns are known to contain sequences for transcription and splicing regulation, which may lead to different mRNA levels and isoforms, and result in modified protein activity (Gasch *et al.*, 1989; Carstens *et al.*, 1998). Alternatively, microsatellite variations could be in linkage disequilibrium with other functional gene variants, and thus indirectly modify gene expression and protein function. TTTA microsatellite in intron 4 has been reported to be in linkage disequilibrium with CT single nucleotide polymorphism (SNP) rs10046 in 3-untranslated region of exon 10 (Kristensen *et*

al., 2000). Linked T-variant and long $(TTTA)_{12}$ allele are associated with elevated aromatase transcript levels in breast cancer tissue (Kristensen *et al.*, 2000).

Two previous studies have also addressed the effect of *CYP19A1* variants on ovarian stimulation outcome. In both of these studies, no genetic interactions were observed between *CYP19A1* C/T SNP (rs10046) and FSH hormone response during ovarian stimulation (de Castro *et al.*, 2004) or the aetiology of severe ovarian hyperstimulation syndrome (Binder *et al.*, 2008). Considering the known linkage between $(TTTA)_n$ microsatellite and C/T SNP, the published studies seemingly contradict the present results. However, genotyping C/T SNP in exon 10 only partially predicts TTTA-repeat length in intron 4 (Kristensen *et al.*, 2000), which makes direct comparisons of study results impossible. In addition, differences in the study populations and ovarian stimulation regimens may account for the discrepancies noted between the clinical outcomes. Intra-cycle GnRH antagonists were used for the rapid down-regulation of pituitary function in all patients, unlike the GnRH agonist long protocol utilized by de Castro and colleagues (de Castro *et al.*, 2004).

Ovarian stimulation outcome was shown to correlate with the follicle count observed in ovaries on ultrasound scan during the preceding early follicular phase of an unstimulated cycle (Gougeon, 1996). Thus, the ovarian follicular response and oocyte maturity in IVF may depend on aromatase gene Ins/ Del and $(TTTA)_n$ genotypes through selective *CYP19A1* gene expression in small antral follicles. In line with this possibility, correlations have been demonstrated between Del allele and shorter TTTA repeat sizes, with smaller ovaries showing fewer antral follicles on days 3–5 of a spontaneous menstrual cycle. Both ovarian size and follicle count are regarded as ovarian reserve markers. Therefore, it is unexpected that *CYP19A1* gene variants would not demonstrate any association with serum FSH

concentration, which is probably the most acknowledged marker of ovarian senescence and responsiveness.

Multiple factors govern ovarian response, along with the most prominent negative effect of increased patient age (Kligman and Rosenwaks, 2001). A previous study suggested that serum anti-FSH antibodies are associated with poor ovarian response to FSH stimulation in IVF, with a potential local antagonizing effect in maturing follicles (Haller *et al.*, 2008). Diminished response to FSH stimulation is also associated with decreased granulosa cell aromatase activity (Hurst *et al.*, 1992) and lower follicular fluid oestradiol concentration (Bahçeci *et al.*, 2007). However, aromatase mRNA and protein levels in granulosa cells in respect to ovarian FSH-sensitivity are not known.

Genetic studies should provide the basis for the pharmacogenetic approach to ovarian stimulation as has recently been demonstrated for patients with unfavourable *FSHR* genotype using higher initial and total FSH doses to overcome relative ovarian insensitivity (Behre *et al.*, 2005). However, whether or not the aromatase gene variants have enough influence on ovarian stimulation outcome in order to be applicable in determining FSH doses in hormonal stimulation remains a challenge for future studies.

Serum oestradiol concentration during ovarian stimulation represents the sum of oestradiol production of all growing follicles. It was found that women with the Del homozygous genotype have higher values of serum oestradiol and oestradiol per follicle punctured. This finding apparently contradicts decreased FSH sensitivity observed in Del/Del patients compared with women carrying at least one Ins allele. However, the Del variation with accompanying shorter TTTA- repeats is associated with smaller ovaries exhibiting fewer antral follicles at the beginning of the natural cycle. The aim of ovarian stimulation is to produce the maximum number of high quality oocytes with the utmost developmental

capacity to sustain fertilization, implantation and pregnancy. Ovarian stimulation is closely followed with monitoring of follicular development two or three times during the stimulation. This vigilance leads to the appropriate adjustments of FSH doses guided by the ultrasound images of the ovaries. Considering the increased doses of FSH required per maturing follicle, it is postulated that the higher serum oestradiol concentrations demonstrated in Del/Del patients might be explained with the exaggerated oestradiol production caused by the excessive FSH stimulation of follicles.

Although no genetic influences on pregnancy outcome were detected, such predictions may not be meaningful, as aromatase is not expressed in the normal endometrium (Kitawaki *et al.*, 1997). Alternatively, the present negative finding can also be a result of the low level of statistical power to reveal minor differences between study groups with insufficient size. Indeed, post-hoc analysis indicated the unsatisfactory power to conclusively prove the absence of a relationship between *CYP19A1* variants and IVF pregnancy success. Intriguingly, in this context, aromatase inhibitors and lower concentrations of oestrogens may contribute to better implantation potential by improving endometrial development, without having a negative anti-oestrogenic effect on folliculogenesis (Verpoest *et al.*, 2006).

Literature offers lines of evidence on the overlapping patient profiles of folliculogenesis abnormalities in women with endometriosis and unexplained infertility, as extensively reviewed by Cahill and Hull (2000). Furthermore, although diagnostic laparoscopy is included in the routine evaluation of female infertility, endometriosis can be underestimated due to the non-visible precursor stages of endometriotic lesions, ending up with misdiagnosis of unexplained infertility. Although the correlations between *CYP19A1* variants and the occurrence of endometriosis and unexplained infertility are presented, the limited size of both patient groups merits consideration of the present findings. Earlier studies have failed to

obtain evidence on the significance of shorter *CYP19A1* TTTA-repeats in the increased risk of endometriosis (Kado *et al.*, 2002; Hur *et al.*, 2007). However, a study of Japanese endometriosis patients showed a preponderance of Del/Del genotype among these patients compared with controls (Kado *et al.*, 2002). This is relevant because Del variant is known to be in strong linkage disequilibrium with short $(TTTA)_7$ allele (Probst-Hensch *et al.*, 1999), which supports the view of low-repeat-number TTTA-alleles as susceptibility factors for endometriosis. In addition, a study conducted among the Greek population suggests *CYP19A1* $(TTTA)_{10}$ allele is consistent with the endometriosis phenotype (Arvanitis *et al.*, 2003). However, this inference has been undermined in a recent meta-analysis that attributes the association to chance (Guo, 2006).

In conclusion, future studies are clearly needed to confirm these preliminary results on the importance of aromatase gene variants in the aetiology of female infertility and ovarian stimulationoutcome.

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Table 1. Associations between *CYP19A1* Ins/Del genotypes and parameters (mean ± SD) describing ovarian stimulation outcome from linear regression analysis adjusted for patient age. Del/Del patients were used as controls.

Parameter	*Del/Del* *Value*	*Ins/Del* *Value*	r	P-*value*	*Ins/Ins* *Value*	r	P-*value*
Age (years)	33.5 ± 6.2	34.0 ± 4.9	–	–	34.0 ± 4.6	–	–
Serum oestradiol (pmol/l)[a]	5485.6 ± 7658.8	4317.1 ± 4707.0	−1146.00	NS	3674.6 ± 3485.9	−1787.29[b]	NS[b]
No. of punctured follicles	12.4 ± 7.6	14.5 ± 6.8	2.32	NS	15.0 ± 6.1	2.85	NS
Oestradiol (pmol/l) per punctured follicle	405.8 ± 293.2	296.1 ± 207.9	−119.45	NS	267.3 ± 306.8	−149.00[c]	NS[c]
Follicular fluid oestradiol (nmol/l)	3859.4 ± 6527.8	2275.3 ± 8870.1	−1773.15	NS	2153.6 ± 4221.2	−1910.23	NS
No. of oocytes	10.1 ± 6.6	13.0 ± 6.7	3.04	NS	12.4 ± 6.3	2.52	NS
Oestradiol (pmol/l) per oocyte	506.9 ± 393.8	356.6 ± 274.8	−169.17	NS	396.0 ± 747.2	−130.78	NS
No. of mature oocytes	8.3 ± 5.8	10.7 ± 5.6	2.58	NS	10.0 ± 5.5	1.94	NS
No. of embryos	6.1 ± 3.9	7.5 ± 4.3	1.49	NS	6.9 ± 3.9	0.94	NS
No. of good-quality embryos	2.5 ± 2.6	3.5 ± 3.2	0.98	NS	2.7 ± 2.4	0.21	NS
Ovarian stimulation duration (days)	9.7 ± 0.8	9.6 ± 0.7	−0.09	NS	9.6 ± 0.8	−0.09	NS
Total FSH (IU) used	1871.7 ± 534.3	1888.2 ± 470.0	−5.15	NS	1904.4 ± 490.5	9.01	NS
FSH (IU) per day	192.7 ± 49.4	195.6 ± 40.0	0.81	NS	197.2 ± 40.1	2.15	NS
FSH (IU) per punctured follicle	262.3 ± 259.0	180.4 ± 146.7	−89.67	0.024	169.8 ± 133.7	−101.27	0.011
FSH (IU) per oocyte	323.4 ± 293.1	229.1 ± 234.7	−104.38	NS	229.4 ± 202.9	−105.03	NS
FSH (IU) per mature oocyte	496.8 ± 568.0	266.6 ± 242.4	−240.84	0.004	294.9 ± 271.4	−211.61	0.012
FSH (IU) per embryo	406.6 ± 305.9	389.0 ± 380.8	−51.81	NS	394.9 ± 342.9	−45.32	NS
FSH (IU) per good-quality embryo	657.4 ± 468.5	782.5 ± 618.2	−9.60	NS	931.8 ± 801.7	120.30	NS

r = regression coefficient of linear regression analysis; NS = not statistically significant.
[a]On day of ovarian puncture; [b]*r* = −2580.60 (*P* = 0.062) if adjusted for ovarian volume; [c]*r* = -173.90 (*P* = 0.032) if adjusted for ovarian volume.

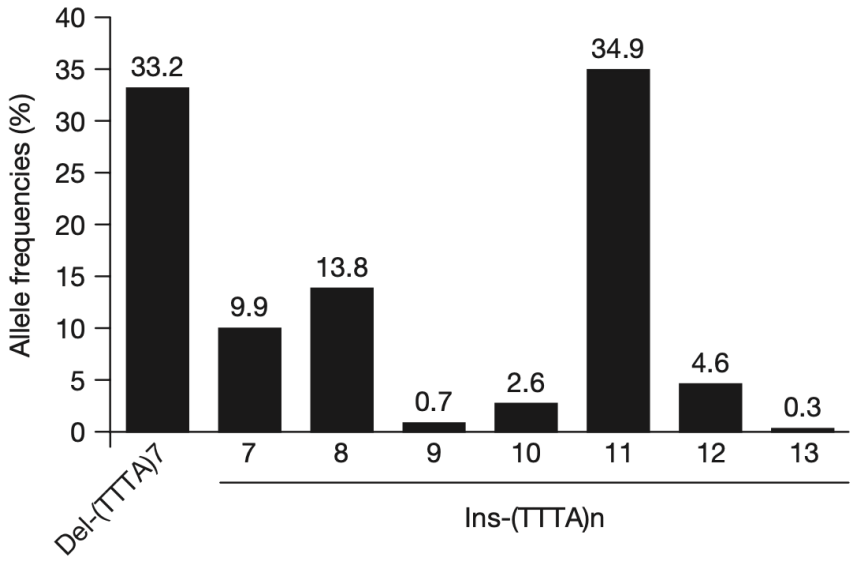

Figure 1. The distribution of *CYP19A1* $(TTTA)_n$ and TCT insertion/deletion (Ins/Del) polymorphism allele frequencies (%) among all IVF patients studied.