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Allelic estrogen receptor 1 (*ESR1*) gene variants predict the outcome of ovarian stimulation in *in vitro* fertilization

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The outcome of *in vitro* fertilization (IVF) depends substantially on the effectiveness of controlled ovarian hyperstimulation (COH) induced by administration of follicle-stimulating hormone (FSH). In COH, endogenously produced estrogens extend the action of FSH in stimulating folliculogenesis. We determined the associations between genetic variations in estrogen receptor *ESR1* and *ESR2* genes and etiology of female infertility, and analysed the influence of these variations on COH outcome—the quantity and quality of oocytes retrieved. *ESR1* PvuII T/C (rs2234693) and XbaI A/G (rs9340799) single-nucleotide polymorphisms (SNPs) and $(TA)_n$ microsatellite polymorphism, as well as *ESR2* RsaI G/A (rs1256049) SNP and $(CA)_n$ microsatellite polymorphism were genotyped in 159 IVF patients. The ovarian response to FSH was diminished in patients with endometriosis when compared to tubal factor infertility. *ESR1* PvuII and XbaI as well as *ESR2* RsaI SNPs were associated with the microsatellite length of the respective genes. Shorter *ESR1* $(TA)_n$ was linked with a higher risk for unexplained infertility, whereas longer *ESR1* $(TA)_n$ associated with PvuII*C allele were predictive of a better COH, but not clinical pregnancy outcome in an age-independent manner. These data suggest the variations in *ESR1* gene, in addition to the age of a woman, may predict the COH outcome in IVF.

Keywords: estrogen receptor gene/*in vitro* fertilization/controlled ovarian hyperstimulation

Introduction

In vitro fertilization (IVF) is the most successful treatment for various causes of infertility. In IVF, multiple follicles are induced to mature by administration of follicle-stimulating hormone (FSH) in a procedure known as controlled ovarian hyperstimulation (COH). The pregnancy outcome of IVF depends substantially on the effectiveness of COH, as measured by the quantity and quality of oocytes retrieved. The ovarian response of IVF patients to FSH stimulation varies considerably and is influenced negatively by increased age of the woman and by reduced ovarian reserve (Kligman and Rosenwaks, 2001). In addition, suboptimal ovarian response to exogenous FSH may be caused by elevated levels of gonadal and gonadotrophin autoantibodies (Meyer *et al.*, 1990).

Estrogens extend the action of FSH on granulosa cells by promoting their proliferation and increasing their expression of FSH receptors (Ireland and Richards, 1978). Estrogen signalling is mediated via binding to estrogen receptors (ERs), which are ligand-dependent transcription factors. Two ERs subtypes exist in humans, i.e. ERα (Walter *et al.*, 1985) and ERβ (Mosselman *et al.*, 1996), coded by *ESR1* and *ESR2* genes, respectively. Gene *ESR1* is located on chromosome 6q25.1 and consists of 8 exons spanning >140 kb; and the *ESR2* (40 kb) maps on chromosome 14q23.2 and is comprised of 8 exons.

In the ovary, ERα is mostly located in the thecal layer, whereas ERβ can be found in granulosa cells of growing follicles at all developmental stages (Pelletier and El-Alfy, 2000). The allocation of different ER subtypes into separate follicular compartments is concordant with the view that the effects of estrogens in folliculogenesis are mediated via the actions of ERα and ERβ on thecal and granulosa cells, respectively. ERs additionally play an essential role in preparing the endometrium for embryo attachment and implantation, with both ERα and ERβ present in all major uterine cell types throughout the menstrual cycle (Matsuzaki *et al.*, 1999).

ER genes harbour several DNA sequence variations that may influence the risk for certain infertility-associated gynaecological disorders and IVF outcome. The *ESR1* intron 1 contains two single-nucleotide polymorphisms (SNPs) at the PvuII (T/C) and XbaI (A/G) restriction sites. The *ESR1* PvuII locus is reportedly associated with the susceptibility to endometriosis (Hsieh *et al.*, 2007) and COH/pregnancy outcome of IVF (Georgiou *et al.*, 1997; Sundarrajan *et al.*, 1999). An additional *ESR1* promoter $(TA)_n$ dinucleotide repeat polymorphism is suggested to increase the risk of premature ovarian failure (Syrrou *et al.*, 1999) and endometriosis (Georgiou *et al.*, 1999). Furthermore, the *ESR2* RsaI (G/A) locus is linked to ovulatory dysfunction of unknown etiology (Sundarrajan *et al.*, 2001).

These previous findings suggest that improving our understanding of ER gene polymorphisms may be important for advancing infertility diagnoses and treatments. Therefore, the purpose of the present study was to determine the importance of *ESR1* PvuII, XbaI and $(TA)_n$, and of *ESR2*

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RsaI and $(CA)_n$ variations in the etiology of female infertility, as well as their contributions to the COH and pregnancy outcome of IVF.

Materials and Methods

Patients

The Ethics Committee of the University of Tartu approved the study and informed consent was obtained from all participants. A total of 159 normally ovulating female patients (mean age 34.1 ± 4.9 years, mean ± SD) who underwent IVF at Nova Vita Clinic in Estonia between July 2004 and December 2005 participated in the study. All patients were infertile for at least one year before entering the study. Their indications for IVF were as follows: tubal factor infertility (reference group, 44.7%, $n = 71$), male factor infertility (30.8%, $n = 49$), endometriosis (9.4%, $n = 15$), unexplained infertility (9.4%, $n = 15$) and infertility due to other reasons such as uterine myomas (5.7%, $n = 9$) (Haller *et al.*, 2007). The mean age of the patients in the groups of male factor infertility, endometriosis and unexplained infertility were similar to that of women in the reference group with tubal factor infertility. Only women with infertility due to other reasons were significantly older (39.2 ± 6.7 and 33.6 ± 3.9 years, respectively, regression coefficient of linear regression analysis $r = 5.7$, $P < 0.001$) than those of reference group.

Serum FSH levels (9.2 ± 5.2 IU/l) were measured for all participants between day 3 and 5 of the spontaneous menstrual cycle using chemiluminescence immunoassay (Immulite 2000® station, Diagnostic Products Corporation, Los Angeles, CA, USA). The patients with other reasons for infertility had significantly elevated FSH levels at day 3–5 of their spontaneous menstrual cycles (14.1 ± 8.4 and 8.6 ± 5.4 IU/l, respectively, $r = 5.0$, $P = 0.005$) when compared to the reference group.

Transvaginal ultrasound scanning of ovaries was performed during the first 5 days of their spontaneous menstrual cycles. Ovarian volume (5.0 ± 2.2 cm^3) was estimated according to the following formula: $1/2(A \times B \times C)$, where A is the longitudinal diameter, B the anteroposterior diameter and C the transverse diameter of the ovary (Sample *et al.*, 1977). The number of small antral follicles (4.5 ± 1.5) was established by ultrasound scanning of both ovaries in longitudinal cross-section. Mean ovarian volume and follicle number were calculated as the sum of values determined for the left and right ovaries divided by two.

COH and IVF

COH was conducted according to the GnRH antagonist protocol. All patients started COH with injection of recombinant FSH (rFSH; Gonal-F, Serono, Rome, Italy) on day 1–3 of menses, continuing daily for 9.6 ± 0.7 days until one day before human chorionic gonadotrophin (hCG) (Ovitrelle, Serono, Rome, Italy) administration. The COH follow-up included 3–4 ultrasound assessments of endometrium and follicular growth. Daily GnRH antagonist administration (0.25 mg) (Cetrotide, Serono, Rome, Italy or Orgalutran, N.V. Organon, Oss, The Netherlands) was initiated if at least one follicle reached the size of ≥14 mm. The GnRH antagonists were given for 4–5 days up to and including the day of hCG administration. Final follicular maturation was achieved using 250 μg of hCG followed by oocyte retrieval 36 h later. Serum estradiol levels (E_2) were measured on the day of oocyte retrieval using chemiluminescence immunoassay (Immulite 2000® station, Diagnostic Products Corporation).

Patients who received IVF (66/159, 41.5%) and ICSI (93/159, 58.5%) performed as in (Salumets *et al.*, 2003) were both included. A maximum of 2 day two embryos were transferred into the uterus, with vaginal progesterone (Lugesteron, Leiras, Turku, Finland) used for luteal support. A positive serum hCG test (≥10 IU/l) conducted 14 days after embryo transfer confirmed pregnancy. The clinical pregnancy was documented by the presence of gestational sac(s) with fetal heartbeat on transvaginal sonography at 6–7 weeks of gestation. Patients with cancelled COH and oocyte fertilization failure were excluded from the study.

Parameters describing COH and clinical pregnancy outcome of IVF

The outcome of COH was determined by multiple parameters. The total dose of FSH used, the number of follicles punctured at oocyte retrieval (follicles) and the number of cumulus–oocyte complexes obtained by oocyte retrieval (oocytes) were counted for all patients. The number of mature oocytes was calculated for both IVF and ICSI patients. The maturity of IVF oocytes was assessed one day after insemination by counting the fertilized and unfertilised meiosis II (MII) oocytes. ICSI oocytes were considered mature if they reached MII stage by 4–6 h after oocyte retrieval. The total number of embryos was calculated by counting the embryos with 2 pronuclei (2PN-embryos). Embryos with at least 4 blastomeres and <20% fragmentation on day 2 after insemination or ICSI were classified to have good quality.

The following parameters were calculated from the total amount of FSH used for COH to determine the amount of FSH used: (i) to mature one follicle, (ii) to obtain one oocyte, (iii) per mature oocyte, (iv) per 2PN-embryo and (v) per good-quality embryo. In addition, serum E_2 and the amount of serum E_2 per follicle were included in the estimation of COH efficiency. The clinical pregnancy rate was calculated by dividing the number of clinical pregnancies with the total number of embryo transfers.

Single nucleotide polymorphism genotyping

Genomic DNA was extracted from peripheral EDTA-blood using the salting-out method (Aljanabi and Martinez, 1997). Patients were genotyped for PvuII (T/C, rs2234693) and XbaI (A/G, rs9340799) SNPs in *ESR1* intron 1, as well as for the RsaI (G/A, rs1256049) SNP in *ESR2* exon 5, using restriction fragment length polymorphism (RFLP) analysis. For the *ESR1* PvuII and XbaI SNPs, the forward and reverse primers were: 5′-CTGCCACCCTATCTG TATC-3′ and 5′-ACCCTGGCGTCGATTATCTG-3′, respectively. For the *ESR2* RsaI SNP, the forward and reverse primers were: 5′-TCTTGCTTTCC CCAGGCTTT-3′ and 5′-ACCTGTCCAGAACAAGATCT-3′, respectively. Amplification of the DNA (50 ng) was performed in a total volume of 15 μl, containing 0.25 μM dNTP-s (MBI Fermentas, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 1× PCR buffer (Solis BioDyne, Tartu, Estonia), 10 pmol of primers (Metabion, Martinsried, Germany) and 1 U HotStart thermostable DNA polymerase HotFirePol® (Solis BioDyne). PCR was performed using Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The reactions were initiated with the DNA denaturation and enzyme activation at 96°C (10 min), followed by 35 cycles of denaturation at 96°C (30 s), annealing at respective temperature for 30 s (56°C for *ESR1* SNPs and 60°C for *ESR2* SNP), elongation at 72°C (1 min) and final extension at 72°C (5 min). All PCR products were visualized under UV light using ethidium bromide staining after electrophoresis in 1.5% agarose gel in 0.5 Tris-borate-EDTA (TBE) buffer.

The PCR products were digested with 5 U of respective restriction enzyme at 37°C for at least 3 h: *ESR1* rs2234693 SNP with PvuII (T, cuttable allele with restriction fragments of 935 and 426 bp; and C, uncuttable allele of 1361 bp), *ESR1* rs9340799 SNP with XbaI (A, cuttable allele with restriction fragments of 980 and 381 bp; and G, uncuttable allele of 1361 bp) and *ESR2* rs1256049 SNP with RsaI (G, uncuttable allele of 156 bp; and A, cuttable allele with restriction fragments of 110 and 46 bp). The DNA restriction fragments were visualized under UV light on 2% agarose gel with ethidium bromide staining. DNA sequencing using an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems, Forster City, CA, USA) was carried out in 5% of the samples to confirm the genotypes obtained by PCR-RFLP method.

Microsatellite genotyping

The $(TA)_n$ microsatellite polymorphism in the *ESR1* promoter region and the $(CA)_n$ microsatellite in *ESR2* intron 5 were genotyped. For the *ESR1* $(TA)_n$ microsatellite amplification, the forward and reverse primers were: 5′-AGACGCATGATATACTTCACC-3′ and TAMRA-5′-CCTACAACTCGA TCTTCTCG-3′, respectively. For the *ESR2* $(CA)_n$ microsatellite amplification, the forward and reverse primers were: 6FAM-5′-GAGGTAAACCAT GGTCTGTACC-3′ and 5′-GTTGAATGAGTGGGCCTCCCT-3′, respectively. PCR was performed, as described above (annealing temperature at 60°C), and the fluorescence-labelled PCR products were analysed for size using an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems). The sizes of the PCR products were determined by Genescan 2.1 software (PE Applied Biosystems). Rox 500 (PE Applied Biosystems) was used as an internal size standard.

Statistical analysis

The R2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA) was used for chi-square tests, Pearson's linear correlations and

linear and logistic regression analysis. Women with tubal factor infertility were used as a reference group. The *ESR1* PvuII TT and XbaI AA homozygotes, as well as *ESR2* RsaI GG homozygotes were used as the baseline genotypes. Statistical significance was set at $P < 0.05$ in all cases.

Results

Characteristics of COH outcome

An average of, 1909.1 ± 503.3 IU of FSH was used during ovarian stimulation. The mean numbers of follicles, oocytes and mature oocytes per patient were 14.0 ± 6.6, 12.1 ± 6.6, and 9.9 ± 5.5, respectively. The study patients had an average of 6.9 ± 4.0 2PN-embryos, 42.1 ± 29.3% of them developed into good-quality day 2 embryos. The mean dose of FSH administered was 200.4 ± 206.5 IU per follicle, 254.4 ± 255.5 IU per retrieved oocyte, 324.4 ± 339.7 IU per mature oocyte, 393.9 ± 342.6 IU per 2PN-embryo and 866.0 ± 696.0 IU per good-quality embryo. The mean serum E_2 and E_2 per follicle were 4235.0 ± 5090.1 and 305.9 ± 294.7 pmol/l, respectively.

Clinical parameters influencing COH outcome

Age adjusted linear regression model revealed, that both the number of follicles detected on day 3–5 of the patient's spontaneous menstrual cycle and the age of a patient were important predictors of the total dose of FSH used in COH. Every additional day 3–5 follicle was associated with 147.8 less units of FSH used in COH ($r = -147.8$, $P < 0.0001$), while a year increased the total amount of FSH in stimulation by 30.8 units ($r = 30.8$, $P < 0.0001$). The number of follicles at oocyte retrieval correlated positively with the follicle count at the early follicular phase of a patient's spontaneous menstrual cycle ($r = 0.55$, $P < 0.00001$) and decreased as the women aged ($r = -0.1$, $P = 0.004$). Similarly, increasing female age negatively correlated with the numbers of retrieved oocytes ($r = -0.4$, $P = 0.002$), mature oocytes ($r = -0.3$, $P = 0.003$) and 2PN-embryos ($r = -0.2$, $P = 0.030$). In addition, as the women aged, the amount of FSH required to obtain one follicle ($r = 17.6$, $P < 0.00001$), one oocyte ($r = 20.2$, $P < 0.00001$), a mature oocyte ($r = 20.7$, $P < 0.001$), a 2PN-embryo ($r = 23.1$, $P < 0.0001$) and a good-quality embryo ($r = 45.4$, $P < 0.001$) were increased.

As the FSH stimulation parameters are age-sensitive, they were analysed using age-adjusted linear regression models. Patients with endometriosis tended to have fewer follicles at oocyte retrieval (10.8 ± 6.2 and 14.8 ± 6.9, respectively, $r = -3.5$, $P = 0.092$) and embryos (4.7 ± 4.1 and 7.6 ± 4.1, respectively, $r = -2.7$, $P = 0.054$) than those of the reference group of tubal factor infertility. It was additionally revealed that significantly more FSH was needed to mature one follicle in women with endometriosis (371.0 ± 466.8 IU FSH per follicle, $r = 183.7$, $P < 0.001$) and with infertility due to other reasons (409.4 ± 312.8 IU FSH per follicle, $r = 153.4$, $P = 0.020$) than that of reference group (171.5 ± 135.7 IU FSH per follicle,). Patients with endometriosis also needed more FSH to get one oocyte, one mature oocyte and one 2PN-embryo ($r = 211.7$, $P = 0.001$; $r = 306.3$, $P < 0.001$ and $r = 326.6$, $P = 0.002$, respectively).

Associations between ER gene SNPs and biallelic means of microsatellites

The distribution of *ESR1* PvuII genotypes among IVF patients was as follows: 25.5% (38/149) were homozygous for TT, 53.7% (80/149) were TC and 20.8% (31/149) were homozygous for CC, with T and C allele frequencies of 52.3 and 47.7%, respectively. The prevalence of *ESR1* XbaI genotypes were: AA 40.3% (60/149), AG 47.0% (70/149) and GG 12.7% (19/149), with A and G allele frequencies of 63.8 and 36.2%, respectively. The *ESR2* RsaI genotypes distributed as follows: 85.5% (130/152) were GG, 13.8% (21/152) were GA and one person (0.7%, 1/152) had AA genotype, which gave the G and A allele frequencies of 92.4 and 7.6%, respectively. Biallelic means of *ESR1* $(TA)_n$ and *ESR2* $(CA)_n$ microsatellites were 17.2 ± 3.0 and 20.7 ± 1.6 repeats, respectively.

The biallelic means of *ESR1* $(TA)_n$ and *ESR2* $(CA)_n$ microsatellites in respect to the *ESR1* and *ESR2* SNPs are shown in Table 1. Linear regression analyses revealed significant associations between *ESR1* $(TA)_n$ biallelic means and the genotypes of *ESR1* PvuII and XbaI restriction sites. The length of *ESR1* $(TA)_n$ increased in the order of PvuII TT → TC → CC genotypes and XbaI AA → AG → GG genotypes. The length of *ESR2* $(CA)_n$ microsatellite was associated with the *ESR2* RsaI genotypes, as the longest *ESR2* $(CA)_n$ sequence was observed in patients having GG genotype and decreased significantly in patients with GA and AA genotypes.

Allelic ER variants and etiology of female infertility

Age-adjusted logistic regression analysis was used to assess the associations between ER gene polymorphisms and the cause of infertility. Genotypes of *ESR1* PvuII and XbaI, *ESR2* RsaI, and the mean values of *ESR1* $(TA)_n$ and *ESR2* $(CA)_n$ repeat polymorphisms in the groups of male factor infertility, endometriosis and infertility due to other reasons were distributed similarly to the reference group of tubal factor infertility. Furthermore, the allele frequencies of *ESR1* PvuII/XbaI and *ESR2* RsaI polymorphic loci were also comparable to the reference across the study groups. However, linear regression model adjusted by the age of the patient showed that unexplained infertility was associated with shorter *ESR1* $(TA)_n$ biallelic mean (15.4 ± 3.1 and 17.3 ± 3.0 repeats, respectively, $r = -1.8$, $P = 0.043$) compared to the reference patients.

Associations of ER gene polymorphisms with COH and pregnancy outcome of IVF

The COH parameters within patient groups with different ER gene polymorphisms are presented in Table 2. In order to assess the associations between gene polymorphisms and the outcome of ovarian stimulation, the linear regression models adjusted by the age of patient were

Table 1: Associations between estrogen receptor gene SNPs and biallelic mean lengths of microsatellites

Genotypes (*n*)	Mean ± SD length of microsatellite	Regression coefficient[a]
Biallelic mean of *ESR1* $(TA)_n$		
ESR1, PvuII		
TT (38)	14.1 ± 2.1	Reference
TC (80)	17.3 ± 2.3	3.2 ($P < 0.00001$)
CC (31)	19.0 ± 2.7	5.7 ($P < 0.00001$)
ESR1, XbaI		
AA (60)	15.7 ± 2.9	Reference
AG (70)	17.6 ± 2.8	2.0 ($P < 0.001$)
GG (19)	19.2 ± 2.3	3.6 ($P < 0.00001$)
Biallelic mean of *ESR2* $(CA)_n$		
ESR2, RsaI		
GG (130)	21.0 ± 1.4	Reference
GA (21)	18.7 ± 0.8	−2.3 ($P < 0.00001$)
AA (1)	15.0[b]	−6.0 ($P < 0.00001$)

[a]Biallelic means of *ESR1* $(TA)_n$ and *ESR2* $(CA)_n$ were compared between different SNP genotypes of *ESR1* and *ESR2* genes by using linear regression models; [b]only one person (absolute value provided); $P < 0.05$ was considered as statistically significantly different; SD, standard deviation.

Table 2: Associations between variations of estrogen receptor genes and parameters (means ± SD) describing the outcome of COH

	Genotypes of *ESR1*, PvuII			Genotypes of *ESR1*, XbaI			Genotypes of *ESR2*, RsaI		
	TT ($n = 38$)[a]	TC ($n = 80$)	CC ($n = 31$)	AA ($n = 60$)[a]	AG ($n = 70$)	GG ($n = 19$)	GG ($n = 130$)[a]	GA ($n = 21$)	AA ($n = 1$)[b]
Total amount of FSH (IU) used for COH	1867.8 ± 521.7	1971.6 ± 499.2	1733.9 ± 395.7	1920.4 ± 491.6	1907.0 ± 521.5	1772.4 ± 366.8	1891.7 ± 497.2	1896.4 ± 432.2	2325.0
Number of follicles after COH	12.9 ± 6.4	14.1 ± 6.6	15.8 ± 6.4 [c]	14.1 ± 6.3	13.8 ± 6.8	15.5 ± 6.5	14.3 ± 6.6	13.7 ± 6.6	8.0
Number of oocytes	11.2 ± 6.2	12.3 ± 6.9	13.4 ± 6.3 [c]	12.4 ± 6.5	11.8 ± 6.8	13.1 ± 6.3	12.2 ± 6.5	12.8 ± 7.2	8.0
Number of mature oocytes	9.3 ± 4.9	10.0 ± 5.9	10.7 ± 5.4	10.3 ± 5.5	9.5 ± 5.6	10.4 ± 5.4	10.0 ± 5.7	10.2 ± 4.6	8.0
FSH (IU) per follicle	205.5 ± 171.4	203.5 ± 179.3	143.6 ± 103.3	183.3 ± 146.2	210.2 ± 191.0	150.0 ± 107.8	185.2 ± 151.1	219.3 ± 229.1	290.6
FSH (IU) per oocyte	247.7 ± 202.4	266.2 ± 257.2	179.9 ± 136.4	222.1 ± 181.1	280.6 ± 272.6	180.2 ± 121.9	240.7 ± 219.5	249.4 ± 254.0	290.6
FSH (IU) per mature oocyte	285.0 ± 224.6	345.8 ± 355.3	249.8 ± 254.6	288.3 ± 303.8	340.0 ± 314.6	277.5 ± 303.7	311.0 ± 303.1	294.1 ± 336.8	290.6
FSH (IU) per 2PN-embryo	422.8 ± 309.4	419.3 ± 406.9	320.8 ± 201.6	378.2 ± 280.6	446.8 ± 430.2	303.1 ± 166.4	410.0 ± 365.8	323.3 ± 191.8	332.1
FSH (IU) per good-quality embryo	1078.2 ± 703.8	727.4 ± 649.3[c]	838.1 ± 716.5[c]	907.9 ± 644.5	801.2 ± 717.2	854.9 ± 793.2	859.9 ± 700.5	846.5 ± 670.4	775.0
E_2 (pmol/l)	3641.4 ± 4015.8	3961.6 ± 4592.0	5971.4 ± 7505.4	3876.9 ± 3703.7	3848.0 ± 4642.4	7361.2 ± 9412.2[c]	4267.5 ± 5203.7	4646.1 ± 5302.0	2415.0
E_2 (pmol/l) per follicle	286.1 ± 222.5	294.1 ± 298.6	367.0 ± 388.7	278.4 ± 198.0	296.4 ± 312.0	441.6 ± 482.3[c]	303.9 ± 301.3	336.5 ± 302.3	301.9
Frequency (%) of clinical pregnancies	24.3	35.5	32.1	22.4	38.8	37.5	32.3	31.6	0

[a]Reference genotypes; [b]only one person (absolute value provided); [c]COH parameters in different *ESR1* and *ESR2* genotypes were compared to the parameters in the reference genotype by using linear regression analysis adjusted by the age of the patient and genotypes studied, the associations between the estrogen receptor gene variants and the occurrence of pregnancy were assessed by the logistic regression models adjusted by age, cause of infertility and procedure (IVF or ICSI) used for oocyte fertilization; $P < 0.05$ was considered statistically significant. Regression coefficients and P-values are given in the text.

used. On average, 5.3 more follicles developed after ovarian stimulation in patients with the *ESR1* PvuII CC genotype ($r = 5.3$, $P = 0.033$) than those of carrying the TT genotype. Similarly, the *ESR1* PvuII CC genotype tended to be associated with a higher number of retrieved oocytes ($r = 5.1$, $P = 0.052$) and 2364.2 pmol/l higher level of E_2 ($r = 2364.2$, $P = 0.070$) compared to the reference TT genotype. The C allele of *ESR1* PvuII (TC and CC genotypes) was also associated with a smaller amount of FSH needed per good-quality embryo ($r = -266.2$, $P = 0.044$). IVF patients carrying the *ESR1* XbaI GG genotype showed significantly increased levels of E_2 ($r = 3526.0$, $P = 0.013$) and E_2 per follicle ($r = 189.0$, $P = 0.019$) than those with the reference AA genotype. However, the *ESR2* RsaI genotype was not associated with the parameters describing ovarian stimulation outcome.

The linear regression model adjusted by patient age and ER gene microsatellite length revealed a positive correlation between the *ESR1* $(TA)_n$ biallelic mean and the number of follicles matured ($r = 0.5$, $P = 0.007$), as well as the oocytes obtained ($r = 0.4$, $P = 0.039$). The length of the *ESR2* $(CA)_n$ microsatellite did not correlate with any of the IVF ovarian stimulation parameters.

The mean clinical pregnancy rate for all study patients was 29.6% (47/159) per embryo transfer. The associations between the ER gene variants and the occurrence of clinical pregnancy were assessed by the logistic regression models adjusted by age, cause of infertility and procedure (IVF or ICSI) used for oocyte fertilization. Neither *ESR1* or *ESR2* SNPs nor the *ESR1* $(TA)_n$ or *ESR2* $(CA)_n$ microsatellites predicted the probability for clinical pregnancy per embryo transfer.

Discussion

In the current study, we examined the complex genotype consisting of *ESR1* and *ESR2* gene variations in infertile patients undergoing IVF. We demonstrated that the *ESR1* PvuII T/C and XbaI A/G genotypes, as well as the *ESR2* RsaI G/A SNP were associated with different lengths of microsatellites of the respective genes. In addition, we detected that shorter *ESR1* $(TA)_n$ variations were more common among women with unexplained infertility compared to the reference group of patients with tubal factor infertility. While a longer *ESR1* $(TA)_n$ polymorphism associated with the *ESR1* PvuII*C allele was related to a better COH outcome in an age-independent manner.

Our data reinforce the general consensus that the age of a woman undergoing IVF is an important predictor of COH outcome. Ovarian ageing accompanied by follicle depletion is reported to cause a decreased response to ovulation induction, requiring higher doses of FSH during COH (Kligman and Rosenwaks, 2001). This was also noted in our study, as higher FSH amounts were required to attain an adequate ovarian response if age of the patients increased. In addition, the reduced number of ovarian follicles at the early follicular phase led to fewer follicles after COH. The predictive value of follicle number in the early follicular phase, as detected by ultrasound, on the ovarian responsiveness to FSH in women undergoing IVF treatment has also been shown previously (Tomas *et al.*, 1997). Furthermore, in our study, patients with infertility due to other reasons were significantly older than the reference group. They also showed significantly elevated FSH levels at the early follicular phase of the menstrual cycle and a greater amount of FSH required to achieve polyfolliculogenesis.

Among women with endometriosis, we noted significantly decreased ovarian responsiveness than in age-matched reference group. The decreased ovarian response to FSH stimulation could be associated with surgical manipulations often conducted on the ovaries of these patients. However, our patients with endometriosis did not differ from the reference group in the mean ovarian volume or the mean follicular count at the early follicular phase of a patient's spontaneous menstrual cycle. In addition, the deleterious effect of the conservative surgery performed for endometriomas on the ovarian response to FSH stimulation conducted in IVF has recently been questioned (Donnez *et al.*, 2001). Previous conflicting studies have associated the increased susceptibility to endometriosis both with *ESR1* PvuII*T allele and shorter $(TA)_n$ microsatellites (Georgiou *et al.*, 1999) and *ESR1* PvuII*C allele (Hsieh *et al.*, 2007). We were unable to show associations between ER gene variations and development of endometriosis. However, significantly shorter *ESR1* $(TA)_n$ biallelic means were detected among women with unexplained infertility. Interestingly, a lower repeat number of the $(TA)_n$ tract is reported to occur more frequently among women with premature ovarian dysfunction (Syrrou *et al.*, 1999). While, women with unexplained infertility are a heterogeneous group of patients, our results suggest that variations in the *ESR1* are one susceptibility factor for unspecified infertility. Still, further studies are needed to confirm our findings.

Our data showed clear associations between COH outcome and genetic variability of *ESR1* among IVF patients. The presence of the *ESR1* PvuII*C allele and a longer $(TA)_n$ dinucleotide repeat polymorphism were associated with better COH outcome: more follicles matured, more oocytes obtained, and lower FSH doses required to get one good-quality embryo. These findings are rather substantial, as for example, patients carrying the *ESR1* PvuII CC genotype developed 5.3 more follicles during COH than patients with the same age but *ESR1* PvuII TT genotype. At the same time, the average number of follicles after COH was 14.0 ± 6.6.

The current results from the Estonian population are in concordance with previous studies conducted in other European (Georgiou *et al.*, 1997; de Castro *et al.*, 2004) and Asian (Sundarrajan *et al.*, 1999) populations. In the first study, patients who were homozygous for the *ESR1* PvuII*C allele were associated with improved follicular quality, as judged by the mean ratio of follicles to oocytes obtained after COH in IVF (Georgiou *et al.*, 1997). While in the study by Sundarrajan *et al.* (1999), *ESR1* PvuII CC homozygotes demonstrated higher mean numbers of follicles, oocytes and embryos when compared to patients possessing TT genotype (Sundarrajan *et al.*, 1999). Furthermore, a similar conclusion was reached recently, following the demonstration that the *ESR1* PvuII*C allele frequency is lower among poor (≤ 3 follicles) compared to normal COH responders (de Castro *et al.*, 2004). In addition, we showed the *ESR1* PvuII CC genotype tended to be and *ESR1* XbaI GG genotype was associated with higher estradiol levels achieved during COH. Comparable association between PvuII/XbaI genotypes and serum estradiol levels has also been reported for postmenopausal women (Schuit *et al.*, 2005).

In all previous studies on genetic predictors of COH outcome, a GnRH agonist long protocol was used for ovarian stimulation (Georgiou *et al.*, 1997; Sundarrajan *et al.*, 1999; de Castro *et al.*, 2004). To the best of our knowledge, the current study was the first to evaluate the impact of ER gene variations on COH outcome using rFSH and GnRH antagonists. Although our results, and those of others, suggest the critical role of *ESR1* gene variants in determining COH outcome, the exact functional importance of these non-coding polymorphisms on ER gene/protein function is still unknown. Recently, however, it was speculated that T allele of *ESR1* PvuII locus may eliminate a functional binding site for the myb family of transcription factors (Herrington *et al.*, 2002).

According to our results, the length of the *ESR2* $(CA)_n$ tract was associated with *ESR2* RsaI genotypes—the length of *ESR2* $(CA)_n$ was the longest in the *ESR2* RsaI GG genotype and decreased significantly in GA and AA genotypes. The value of the *ESR2* RsaI SNP has been studied with respect to ovulatory dysfunction, and AA

homozygosity was found to be associated with ovulatory defects of unknown etiology (Sundarrajan *et al.*, 2001). Notwithstanding that the *ESR2* exon 5 RsaI restriction site polymorphism does not lead to amino acid change, it is plausible that it may directly influence *ESR2* gene expression or alternatively could be linked to yet unidentified causative DNA sequence variant. In the current study, we were unable to show any relationship between *ESR2* gene variants and COH outcome.

Contrary to findings in previous studies (Georgiou *et al.*, 1997; Sundarrajan *et al.*, 1999), our results suggested neither *ESR1* nor *ESR2* variants predict the chance for clinical pregnancy per embryo transfer. However, since the polymorphisms in *ESR1* showed an association with the COH outcome, these variations could have impact on the cumulative pregnancy rate per COH, rather than per single embryo transfer. The predictive value of ER gene polymorphisms on cumulative pregnancy outcome needs further evaluation.

We conclude that the genotypes of *ESR1* PvuII and XbaI, as well as *ESR2* RsaI, predict the length of microsatellites of the corresponding genes. Additionally, our results advocate that a shorter *ESR1* $(TA)_n$ microsatellite could be a potential genetic risk factor for unexplained female infertility being also associated with a poorer ovarian response to FSH stimulation. In addition, our results strongly suggest longer *ESR1* $(TA)_n$ microsatellites in association with the *ESR1* PvuII*C allele predict a better COH, but not pregnancy outcome in IVF.

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