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

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17

18 **Abstract**

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

35

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

37

38 **Introduction**

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

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

55 In the ovary, ERa is mostly located in the thecal layer, whereas ERb can be found in
56 granulosa cells of growing follicles at all developmental stages (Pelletier and El-Alfy, 2000).
57 The allocation of different ER subtypes into separate follicular compartments is con- cordant
58 with the view that the effects of estrogens in folliculogenesis are mediated via the actions of
59 ERa and ERb on thecal and granulosa cells, respectively. ERs additionally play an essential
60 role in preparing the endometrium for embryo attachment and implantation, with both ERa

61 and ERb present in all major uterine cell types throughout the menstrual cycle (Matsuzaki et

62 al., 1999).

63 ER genes harbour several DNA sequence variations that may influ- ence the risk for certain

64 infertility-associated gynaecological disorders and IVF outcome. The ESR1 intron 1 contains

65 two single-nucleotide polymorphisms (SNPs) at the PvuII (T/C) and XbaI (A/G) restriction

66 sites. The ESR1 PvuII locus is reportedly associated with the suscep- tibility to endometriosis

67 (Hsieh et al., 2007) and COH/pregnancy outcome of IVF (Georgiou et al., 1997; Sundarrajan

68 et al., 1999). An additional ESR1 promoter $(TA)_n$ dinucleotide repeat polymorph- ism is

69 suggested to increase the risk of premature ovarian failure (Syrrou et al., 1999) and

70 endometriosis (Georgiou et al., 1999). Furthermore, the ESR2 RsaI (G/A) locus is linked to

71 ovulatory dys- function of unknown etiology (Sundarrajan et al., 2001).

72 These previous findings suggest that improving our understanding of ER gene

73 polymorphisms may be important for advancing infertility diag- noses and treatments.

74 Therefore, the purpose of the present study was to determine the importance of ESR1 PvuII,

75 XbaI and $(TA)_n$, and of ESR2 RsaI and $(CA)_n$ variations in the etiology of female infertility,

76 as well as their contributions to the COH and pregnancy outcome of IVF.

77

78 **Materials and Methods**

79 Patients

80 The Ethics Committee of the University of Tartu approved the study and informed consent

81 was obtained from all participants. A total of 159 normally ovulating female patients (mean

82 age 34.1+4.9 years, mean+SD) who under- went IVF at Nova Vita Clinic in Estonia between

83 July 2004 and December 2005 participated in the study. All patients were infertile for at least
84 one year before entering the study. Their indications for IVF were as follows: tubal factor
85 infertility (reference group, 44.7%, n 1⁄4 71), male factor infertility (30.8%, n 1⁄4 49),
86 endometriosis (9.4%, n 1⁄4 15), unexplained infertility (9.4%, n 1⁄4 15) and infertility due to
87 other reasons such as uterine myomas (5.7%, n 1⁄4 9) (Haller et al., 2007). The mean age of
88 the patients in the groups of male factor infertility, endometriosis and unexplained infertility
89 were similar to that of women in the reference group with tubal factor inferti- lity. Only
90 women with infertility due to other reasons were significantly older (39.2 + 6.7 and 33.6 +
91 3.9 years, respectively, regression coefficient of linear regression analysis r 1⁄4 5.7, P , 0.001)
92 than those of reference group.

93 Serum FSH levels (9.2+5.2IU/l) were measured for all participants between day 3 and 5 of
94 the spontaneous menstrual cycle using chemilumines- cence immunoassay (Immulite 2000^{W}
95 station, Diagnostic Products Corporation, Los Angeles, CA, USA). The patients with other
96 reasons for infertility had significantly elevated FSH levels at day 3 – 5 of their spontaneous
97 menstrual cycles (14.1 + 8.4 and 8.6 + 5.4 IU/l, respectively, r 1⁄4 5.0, P 1⁄4 0.005) when
98 compared to the reference group.

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

106 COH and IVF

107 COH was conducted according to the GnRH antagonist protocol. All patients started COH
108 with injection of recombinant FSH (rFSH; Gonal-F, Serono, Rome, Italy) on day 1–3 of
109 menses, continuing daily for 9.6+0.7 days until oneday before human chorionic
110 gonadotrophin (hCG) (Ovitrelle, Serono, Rome, Italy) administration. The COH follow-up
111 included 3 – 4 ultrasound assess- ments of endometrium and follicular growth. Daily GnRH
112 antagonist adminis- tration (0.25 mg) (Cetrotide, Serono, Rome, Italy or Orgalutran, N.V.
113 Organon, Oss, The Netherlands) was initiated if at least one follicle reached the size of 14
114 mm. The GnRH antagonists were given for 4–5 days up to and including the day of hCG
115 administration. Final follicular maturation was achieved using 250 mg of hCG followed by
116 oocyte retrieval 36 h later. Serum estradiol levels (E2) were measured on the day of oocyte
117 retrieval using chemiluminescence immunoassay (Immulite 2000[W] station, Diagnostic
118 Products Corporation).

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

126 Parameters describing COH and clinical pregnancy outcome of IVF

127 The outcome of COH was determined by multiple parameters. The total dose of FSH used,
128 the number of follicles punctured at oocyte retrieval (follicles) and the number of cumulus –

129 oocyte complexes obtained by oocyte retrieval (oocytes) were counted for all patients. The
130 number of mature oocytes was cal- culated for both IVF and ICSI patients. The maturity of
131 IVF oocytes was assessed one day after insemination by counting the fertilized and
132 unfertilised meiosis II (MII) oocytes. ICSI oocytes were considered mature if they reached
133 MII stage by 4 – 6 h after oocyte retrieval. The total number of embryos was calculated by
134 counting the embryos with 2 pronuclei (2PN-embryos). Embryos with at least 4 blastomeres
135 and ,20% fragmentation on day 2 after insemination or ICSI were classified to have good
136 quality.

137 The following parameters were calculated from the total amount of FSH used for COH to
138 determine the amount of FSH used: (i) to mature one follicle, (ii) to obtain one oocyte, (iii)
139 per mature oocyte, (iv) per 2PN-embryo and (v) per good-quality embryo. In addition, serum
140 E2 and the amount of serum E2 per follicle were included in the estimation of COH
141 efficiency. The clinical preg- nancy rate was calculated by dividing the number of clinical
142 pregnancies with the total number of embryo transfers.

143 Single nucleotide polymorphism genotyping

144 Genomic DNA was extracted from peripheral EDTA-blood using the salting- out method
145 (Aljanabi and Martinez, 1997). Patients were genotyped for PvuII (T/C, rs2234693) and XbaI
146 (A/G, rs9340799) SNPs in ESR1 intron 1, as well as for the RsaI (G/A, rs1256049) SNP in
147 ESR2 exon 5, using restriction fragment length polymorphism (RFLP) analysis. For the
148 ESR1 PvuII and XbaI SNPs, the forward and reverse primers were: 5’-
149 CTGCCACCCTATCTG TATC-3’ and 5’ -ACCCTGGCGTCGATTATCTG-3’, respectively.
150 For the ESR2 RsaI SNP, the forward and reverse primers were: 5’-TCTTGCTTTCC
151 CCAGGCTTT-3’ and 5’ -ACCTGTCCAGAACAAGATCT-3’, respectively. Amplification

152 of the DNA (50 ng) was performed in a total volume of 15 ml, containing 0.25 mM dNTP-s
153 (MBI Fermentas, Vilnius, Lithuania), 2.5 mM $MgCl_2$, 1 PCR buffer (Solis BioDyne, Tartu,
154 Estonia), 10 pmol of primers (Metabion, Martinsried, Germany) and 1 U HotStart
155 thermostable DNA poly- merase HotFirePolw (Solis BioDyne). PCR was performed using
156 Eppendorf thermal cycler (Eppendorf, Hamburg, Germany). The reactions were initiated with
157 the DNA denaturation and enzyme activation at 96C (10 min), followed by 35 cycles of
158 denaturation at 96C (30 s), annealing at respective temperature for 30 s (568C for ESR1
159 SNPs and 60C for ESR2 SNP), elongation at 72C (1 min) and final extension at 72C (5 min).
160 All PCR products were visualized under UV light using ethidium bromide staining after
161 electrophoresis in 1.5% agarose gel in 0.5 Tris-borate-EDTA (TBE) buffer.

162 The PCR products were digested with 5 U of respective restriction enzyme at 378C for at
163 least 3 h: ESR1 rs2234693 SNP with PvuII (T, cuttable allele with restriction fragments of
164 935 and 426 bp; and C, uncuttable allele of 1361 bp), ESR1 rs9340799 SNP with XbaI (A,
165 cuttable allele with restriction fragments of 980 and 381 bp; and G, uncuttable allele of 1361
166 bp) and ESR2 rs1256049 SNP with RsaI (G, uncuttable allele of 156 bp; and A, cuttable
167 allele with restriction fragments of 110 and 46 bp). The DNA restriction fragments were
168 visualized under UV light on 2% agarose gel with ethidium bromide staining. DNA
169 sequencing using an ABI Prism 377 automated DNA sequencer (PE Applied Biosystems,
170 Forster City, CA, USA) was carried out in 5% of the samples to confirm the genotypes
171 obtained by PCR-RFLP method.

172 Microsatellite genotyping

173 The $(TA)_n$ microsatellite polymorphism in the ESR1 promoter region and the $(CA)_n$
174 microsatellite in ESR2 intron 5 were genotyped. For the ESR1 $(TA)_n$ microsatellite
175 amplification, the forward and reverse primers were: 5’ -AGACGCATGATATACTTCACC-

176 3' and TAMRA-5' -CCTACAACTCGA TCTTCTCG-3', respectively. For the ESR2 (CA)n

177 microsatellite amplification, the forward and reverse primers were: 6FAM-5'-

178 GAGGTAAACCAT GGTCTGTACC-3' and 5' -GTTGAATGAGTGGGCCTCCCT-3' ,

179 respectively. PCR was performed, as described above (annealing temperature at 608C), and

180 the fluorescence-labelled PCR products were analysed for size using an ABI Prism 377

181 automated DNA sequencer (PE Applied Biosystems). The sizes of the PCR products were

182 determined by Genescan 2.1 software (PE Applied Biosystems). Rox 500 (PE Applied

183 Biosystems) was used as an internal size standard.

184 Statistical analysis

185 The R2.3.1 A Language and Environment (Free Software Foundation, Boston, MA, USA)

186 was used for chi-square tests, Pearson's linear correlations and linear and logistic regression

187 analysis. Women with tubal factor infertility were used as a reference group. The ESR1 PvuII

188 TT and XbaI AA homozygotes, as well as ESR2 RsaI GG homozygotes were used as the

189 baseline genotypes. Statistical significance was set at P , 0.05 in all cases.

190

191 **Results**

192 Characteristics of COH outcome

193 An average of, 1909.1+503.3 IU of FSH was used during ovarian stimulation. The mean

194 numbers of follicles, oocytes and mature oocytes per patient were 14.0 + 6.6, 12.1 + 6.6, and

195 9.9+5.5, respect- ively. The study patients had an average of 6.9+4.0 2PN-embryos,

196 42.1+29.3% of them developed into good-quality day 2 embryos. The mean dose of FSH

197 administered was 200.4 + 206.5 IU per follicle, 254.4 + 255.5 IU per retrieved oocyte, 324.4

198 + 339.7 IU per mature oocyte, 393.9 + 342.6 IU per 2PN-embryo and 866.0 + 696.0 IU per

199 good-quality embryo. The mean serum E_2 and E_2 per follicle were 4235.0 + 5090.1 and

200 305.9+294.7 pmol/l, respectively.

201 Clinical parameters influencing COH outcome

202 Age adjusted linear regression model revealed, that both the number of follicles detected on

203 day 3 – 5 of the patient's spontaneous menstrual cycle and the age of a patient were important

204 predictors of the total dose of FSH used in COH. Every additional day 3–5 follicle was

205 associated with 147.8 less units of FSH used in COH (r 1⁄4 2147.8, P , 0.0001), while a year

206 increased the total amount of FSH in stimu- lation by 30.8 units (r 1⁄4 30.8, P , 0.0001). The

207 number of follicles at oocyte retrieval correlated positively with the follicle count at the early

208 follicular phase of a patient's spontaneous menstrual cycle (r 1⁄4 0.55, P , 0.00001) and

209 decreased as the women aged (r 1⁄4 20.1, P 1⁄4 0.004). Similarly, increasing female age

210 negatively correlated with the numbers of retrieved oocytes (r 1⁄4 20.4, P 1⁄4 0.002), mature

211 oocytes (r 1⁄4 20.3, P 1⁄4 0.003) and 2PN-embryos (r 1⁄4 20.2, P 1⁄4 0.030). In addition, as the

212 women aged, the amount of FSH required to obtain one follicle (r 1⁄4 17.6, P , 0.00001), one

213 oocyte (r 1⁄4 20.2, P , 0.00001), a mature oocyte (r 1⁄4 20.7, P , 0.001), a 2PN-embryo (r 1⁄4

214 23.1, P , 0.0001) and a good- quality embryo (r 1⁄4 45.4, P , 0.001) were increased.

215 As the FSH stimulation parameters are age-sensitive, they were analysed using age-adjusted

216 linear regression models. Patients with endometriosis tended to have fewer follicles at oocyte

217 retrieval (10.8 + 6.2 and 14.8 + 6.9, respectively, r 1⁄4 23.5, P 1⁄4 0.092) and embryos

218 (4.7+4.1 and 7.6+4.1, respectively, r1⁄422.7, P1⁄4 0.054) than those of the reference group of

219 tubal factor infertility. It was additionally revealed that significantly more FSH was needed to

220 mature one follicle in women with endometriosis (371.0 + 466.8 IU FSH per follicle, r 1⁄4

221 183.7, P , 0.001) and with infertility due to other reasons (409.4 + 312.8 IU FSH per follicle,

222 r 1⁄4 153.4, P 1⁄4 0.020) than that of reference group (171.5 + 135.7 IU FSH per follicle,).

223 Patients with endometriosis also needed more FSH to get one oocyte, one mature oocyte and

224 one 2PN-embryo (r 1⁄4 211.7, P1⁄40.001; r1⁄4306.3, P,0.001 and r1⁄4326.6, P1⁄40.002,

225 respectively).

226 Associations between ER gene SNPs and biallelic means of microsatellites

227 The distribution of ESR1 PvuII genotypes among IVF patients was as follows: 25.5%

228 (38/149) were homozygous for TT, 53.7% (80/149) were TC and 20.8% (31/149) were

229 homozygous for CC, with T and C allele frequencies of 52.3 and 47.7%, respectively. The

230 prevalence of ESR1 XbaI genotypes were: AA 40.3% (60/149), AG 47.0% (70/ 149) and GG

231 12.7% (19/149), with A and G allele frequencies of 63.8 and 36.2%, respectively. The ESR2

232 RsaI genotypes distributed as follows: 85.5% (130/152) were GG, 13.8% (21/152) were GA

233 and one person (0.7%, 1/152) had AA genotype, which gave the G and A allele frequencies

234 of 92.4 and 7.6%, respectively. Biallelic means of ESR1 $(TA)_n$ and ESR2 $(CA)_n$

235 microsatellites were 17.2 + 3.0 and 20.7 + 1.6 repeats, respectively.

236 The biallelic means of ESR1 $(TA)_n$ and ESR2 $(CA)_n$ microsatellites in respect to the ESR1

237 and ESR2 SNPs are shown in Table 1. Linear regression analyses revealed significant

238 associations between ESR1 $(TA)_n$ biallelic means and the genotypes of ESR1 PvuII and XbaI

239 restriction sites. The length of ESR1 $(TA)_n$ increased in the order of

240 PvuIITT!TC!CCgenotypesandXbaIAA!AG!GGgeno- types. The length of ESR2 $(CA)_n$

241 microsatellite was associated with the ESR2 RsaI genotypes, as the longest ESR2 $(CA)_n$

242 sequence was observed in patients having GG genotype and decreased significantly in

243 patients with GA and AA genotypes.

244 Allelic ER variants and etiology of female infertility

245 Age-adjusted logistic regression analysis was used to assess the associations between ER
246 gene polymorphisms and the cause of infer- tility. Genotypes of ESR1 PvuII and XbaI, ESR2
247 RsaI, and the mean values of ESR1 $(TA)_n$ and ESR2 $(CA)_n$ repeat polymorphisms in the
248 groups of male factor infertility, endometriosis and infertility due to other reasons were
249 distributed similarly to the reference group of tubal factor infertility. Furthermore, the allele
250 frequencies of ESR1 PvuII/XbaI and ESR2 RsaI polymorphic loci were also comparable to
251 the reference across the study groups. However, linear regression model adjusted by the age
252 of the patient showed that unexplained infertility was associated with shorter ESR1 $(TA)_n$
253 biallelic mean (15.4 + 3.1 and 17.3 + 3.0 repeats, respectively, r 1⁄4 21.8, P 1⁄4 0.043)
254 compared to the reference patients.

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

256 The COH parameters within patient groups with different ER gene polymorphisms are
257 presented in Table 2. In order to assess the associa- tions between gene polymorphisms and
258 the outcome of ovarian stimu- lation, the linear regression models adjusted by the age of
259 patient were used. On average, 5.3 more follicles developed after ovarian stimulation in
260 patients with the ESR1 PvuII CC genotype (r 1⁄4 5.3, P 1⁄4 0.033) than those of carrying the
261 TT genotype. Similarly, the ESR1 PvuII CC genotype tended to be associated with a higher
262 number of retrieved oocytes (r 1⁄4 5.1, P 1⁄4 0.052) and 2364.2 pmol/l higher level of E_2 (r
263 1⁄4 2364.2, P 1⁄4 0.070) compared to the reference TT genotype. The C allele of ESR1 PvuII
264 (TC and CC genotypes) was also associated with a smaller amount of FSH needed per good-
265 quality embryo (r 1⁄4 2266.2, P 1⁄4 0.044). IVF patients carrying the ESR1 XbaI GG
266 genotype showed significantly increased levels of E_2 (r 1⁄4 3526.0, P 1⁄4 0.013) and E_2 per
267 follicle (r 1⁄4 189.0, P 1⁄4 0.019) than those with the reference AA genotype. However, the

268 ESR2 RsaI genotype was not associated with the parameters describing ovarian stimulation
269 outcome.

270 The linear regression model adjusted by patient age and ER gene microsatellite length
271 revealed a positive correlation between the ESR1 $(TA)_n$ biallelic mean and the number of
272 follicles matured (r 1⁄4 0.5, P 1⁄4 0.007), as well as the oocytes obtained (r 1⁄4 0.4, P 1⁄4
273 0.039). The length of the ESR2 $(CA)_n$ microsatellite did not correlate with any of the IVF
274 ovarian stimulation parameters.

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

281

282 **Discussion**

283 In the current study, we examined the complex genotype consisting of ESR1 and ESR2 gene
284 variations in infertile patients undergoing IVF. We demonstrated that the ESR1 PvuII T/C
285 and XbaI A/G genotypes, as well as the ESR2 RsaI G/A SNP were associated with different
286 lengths of microsatellites of the respective genes. In addition, we detected that shorter ESR1
287 $(TA)_n$ variations were more common among women with unexplained infertility compared to
288 the reference group of patients with tubal factor infertility. While a longer ESR1 $(TA)_n$
289 polymorphism associated with the ESR1 PvuII*C allele was related to a better COH outcome
290 in an age-independent manner.

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

304 Among women with endometriosis, we noted significantly decreased ovarian responsiveness
305 than in age-matched reference group. The decreased ovarian response to FSH stimulation
306 could be associated with surgical manipulations often conducted on the ovaries of these
307 patients. However, our patients with endometriosis did not differ from the reference group in
308 the mean ovarian volume or the mean follicular count at the early follicular phase of a
309 patient's spontaneous menstrual cycle. In addition, the deleterious effect of the conservative
310 surgery performed for endometriomas on the ovarian response to FSH stimulation conducted
311 in IVF has recently been ques- tioned (Donnez et al., 2001). Previous conflicting studies have
312 associ- ated the increased susceptibility to endometriosis both with ESR1 PvuII*T allele and
313 shorter $(TA)_n$ microsatellites (Georgiou et al., 1999) and ESR1 PvuII*C allele (Hsieh et al.,
314 2007). We were unable to show associations between ER gene variations and develop- ment
315 of endometriosis. However, significantly shorter ESR1 $(TA)_n$ biallelic means were detected

316 among women with unexplained infer- tility. Interestingly, a lower repeat number of the

317 $(TA)_n$ tract is reported to occur more frequently among women with premature ovarian

318 dysfunction (Syrrou et al., 1999). While, women with unex- plained infertility are a

319 heterogeneous group of patients, our results suggest that variations in the ESR1 are one

320 susceptibility factor for unspecified infertility. Still, further studies are needed to confirm our

321 findings.

322 Our data showed clear associations between COH outcome and genetic variability of ESR1

323 among IVF patients. The presence of the ESR1 PvuII*C allele and a longer $(TA)_n$

324 dinucleotide repeat poly- morphism were associated with better COH outcome: more follicles

325 matured, more oocytes obtained, and lower FSH doses required to get one good-quality

326 embryo. These findings are rather substantial, as for example, patients carrying the ESR1

327 PvuII CC genotype deve- loped 5.3 more follicles during COH than patients with the same

328 age but ESR1 PvuII TT genotype. At the same time, the average number of follicles after

329 COH was 14.0 + 6.6.

330 The current results from the Estonian population are in concordance with previous studies

331 conducted in other European (Georgiou et al., 1997; de Castro et al., 2004) and Asian

332 (Sundarrajan et al., 1999) populations. In the first study, patients who were homozygous for

333 the ESR1 PvuII*C allele were associated with improved follicular quality, as judged by the

334 mean ratio of follicles to oocytes obtained after COH in IVF (Georgiou et al., 1997). While in

335 the study by Sundarrajan et al. (1999), ESR1 PvuII CC homozygotes demonstrated higher

336 mean numbers of follicles, oocytes and embryos when com- pared to patients possessing TT

337 genotype (Sundarrajan et al., 1999). Furthermore, a similar conclusion was reached recently,

338 following the demonstration that the ESR1 PvuII*C allele frequency is lower among poor (3

339 follicles) compared to normal COH responders (de Castro et al., 2004). In addition, we

340 showed the ESR1 PvuII CC geno- type tended to be and ESR1 XbaI GG genotype was
341 associated with higher estradiol levels achieved during COH. Comparable association
342 between PvuII/XbaI genotypes and serum estradiol levels has also been reported for
343 postmenopausal women (Schuit et al., 2005).

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

353 According to our results, the length of the ESR2 $(CA)_n$ tract was associated with ESR2 RsaI
354 genotypes—the length of ESR2 $(CA)_n$ was the longest in the ESR2 RsaI GG genotype and
355 decreased signifi- cantly in GA and AA genotypes. The value of the ESR2 RsaI SNP has
356 been studied with respect to ovulatory dysfunction, and AA homozygosity was found to be
357 associated with ovulatory defects of unknown etiology (Sundarrajan et al., 2001).
358 Notwithstanding that the ESR2 exon 5 RsaI restriction site polymorphism does not lead to
359 amino acid change, it is plausible that it may directly influence ESR2 gene expression or
360 alternatively could be linked to yet uniden- tified causative DNA sequence variant. In the
361 current study, we were unable to show any relationship between ESR2 gene variants and
362 COH outcome.

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

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

375

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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.

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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.