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Original Article

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A Potential Relationship Between Estrogen Receptors Polymorphisms, Sperm Function and *in vitro* Fertilization Success: A Preliminary Study*

Potencjalny związek między polimorfizmami genów receptorów estrogenowych a parametrami nasienia i wynikiem zapłodnienia *in vitro* – badania wstępne

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B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Estrogen receptor 1 (ESR1) and 2 (ESR2) play an important role in regulating fertility in the human reproductive system. Polymorphisms of these receptor genes have been implicated in male infertility in both Chinese and Caucasian populations. However, studies have produced inconsistent results. Spermatozoa defects that result in conception deficiencies could be related to estrogens, their receptors, or genes involved in estrogen-related pathways. This study aims to explore the potential association between the *ESR1* and the *ESR2* polymorphisms in relation to semen parameters of Caucasian males as well as fertilization success.

Materials/Methods: A total of 116 males were included in this study. Forty couples underwent conventional *in vitro* fertilization, while 76 couples were treated by intracytoplasmic sperm injection. Standard semen analyses were performed according to the World Health Organization criteria. Polymerase chain reaction and restriction fragment length polymorphisms were used to determine genotype and allele distributions.

Results: A strong association between the *ESR1* rs2234693 recognized by PvuII enzyme, genotype/allele distribution and fertilization success was shown. The T allele occurrence was significantly lower in the case of fertilization failure ($p = 0.02$). Additionally, the TT genotype was absent in the same group ($p=0.02$). In the case of the remaining analyzed polymorphisms, little to no interdependence of genotype/allele distribution and fertilization success was noted.

Conclusions: Apart from *ESR1* rs2234693, the study failed to demonstrate that fertilization success was associated with the selected polymorphisms. In most cases, we did not discover a relationship between both estrogen receptors polymorphisms and sperm function.

Keywords: male infertility, estrogen receptors, single nucleotide polymorphisms (SNPs), IVF – *in vitro* fertilization, ICSI – intracytoplasmic sperm injection

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Abbreviations: **ESR1** – estrogen receptor 1; **ESR2** – estrogen receptor 2; **FDU** – fast digest unit; **gDNA** – genomic DNA; **GnRH** – gonadotropin-releasing hormone; **HOS** – hypoosmotic test; **HWE** – Hardy-Weinberg equilibrium; **ICSI** – intracytoplasmic sperm injection; **IVF** – in vitro fertilization; **MII** – metaphase II; **NCBI** – National Center for Biotechnology Information; **SNPs** – single nucleotide polymorphisms; **PCR** – polymerase chain reaction; **RFLP** – restriction fragments length polymorphism; **TBE** – tris/boric acid/EDTA; **WHO** – World Health Organization.

INTRODUCTION

Male infertility affects 20% to 25% of couples and in the half cases, conception failures etiology remains idiopathic [26]. It is estimated that approximately 15% of spermatogenic failure and/or sperm dysfunction is the result of gene mutations and chromosomal aberrations [26]. Some genetic disturbances can lead to androgen-estrogen imbalances, resulting in disrupted sperm function and conception problems. Studies on the polymorphic variations of estrogen receptors are becoming more common, but they are insufficient in addressing the genetic changes in estrogens and their receptors concerning the physiology and pathophysiology of the male reproductive system.

Impaired sperm production results from a multitude of factors: oxidative stress, environmental factors, hormonal imbalance, congenital diseases, genetic causes, and a host of other issues. Still, in approximately 50% of male infertility cases, the etiology remains unknown [20, 31].

Estradiol is a key factor for germ cell survival [25], and when production is disturbed, impaired sperm production occurs. Two main estrogen receptor subtypes mediate estrogen-related actions and are present in different stages of human germ cells [7, 8]. In recent years, there has been increasing interest in estrogen signal transduction disorders that may result from genetic polymorphisms in genes encoding estrogen receptors. There have been few studies investigating the direct relationship of these polymorphisms with semen parameters and sperm fertilizing ability. These studies have produced conflicting results. The results may vary due to ethnic differences in the population surveyed, genetic background of the participants, population sample size, or environmental factors or habits [5, 28]. The studies focusing on the ethnic diversity of single nucleotide polymorphisms (SNPs) showed a dependence on the relationship between the genes encoding estrogen receptor SNPs and osteoporosis [22, 38].

Estrogens have come to the forefront for their role in the pathophysiology of male infertility. These steroid hormones are produced in the testis from testosterone through the actions of cytochrome P450, and they act via specific receptors known as estrogen receptor 1 (ESR1) and 2 (ESR2) [11, 18]. Separate genes encode these receptors: the *ESR1* gene is localized on chromosome 6q25.1-q25.2 [14, 27], and the *ESR2* gene is found on chromosome 14q23.2-q23.3 [13, 15]. Estrogen receptors are expressed in almost all tissues of the male reproductive tract, and their expression varies along different stages of development [9, 17]. This suggests an influence of estrogens on the maturation process and function of the male reproductive system. Estrogens, acting through their receptors, regulate Sertoli and Leydig cell numbers and impact spermatogenesis [4, 6]. In animal models, impaired function or knock-out of *ESR1* leads to infertility by reducing seminal fluid resorption and atrophy of the testes. In *ESR1* knock-out mice, lowered mating frequency, inferior sperm parameters, and decreased fertilization potential have been observed [1, 12, 29]. *ESR1* and *ESR2*, as well as aromatase P450, are expressed in male gametes [3, 25, 30], further strengthening a suggested relationship between estrogens and male fertility.

The development and increasing availability of *in vitro* fertilization techniques encourages exploration of factors that may influence their outcome. The influence of estrogens and their receptors on fertilization mechanisms can be observed in conventional *in vitro* fertilization (IVF) vs. the intracytoplasmic sperm injection (ICSI) model. Comparing the presence of ESR genetic polymorphisms with IVF outcomes allows an examination of estrogen action and the fertilization process. According to the literature, two *ESR1* polymorphisms (rs9340799 and rs2234693) were described to have an association with azoospermia and severe oligozoospermia [21, 33]. *ESR2* rs1256049 polymorphism was found to be linked with male infertility and it is probably associated with its influence on luteinizing hormone secretion [2]. The remaining two polymorphisms were suspected to be associated with fertility, but the results were inconclusive.

This study aims to analyze the relationship between *ESR1* and *ESR2* polymorphisms with sperm parameters and fertilization success defined as percentage of fertilized oocytes in both conventional *in vitro* fertilization, and intracytoplasmic sperm injection procedures. Selected *ESR1* polymorphisms: rs9340799 (NC_000006.12:g.151842246A>G) and rs2234693 (NC_000006.12:g.151842200T>C) were recognized by *Xba*I and *Pvu*II restriction enzymes, respectively and *ESR2* polymorphisms: rs1256120 (NC_000014.9:g.64338283T>C), rs1256049 (NC_000014.9:g.64257333C>T) and rs4986938 (NC_000014.9:g.64233098C>T) were recognized by *AlwN*I, *Rsa*I, and *Alu*I, respectively We wanted to analyze the genotype and allele's distribution in patient subgroups with and without normozoospermia, as confirmed by concentration, motility with progressive movement, sperm morphology, and vitality (assessed using hypoosmotic swelling test).

MATERIALS AND METHODS

Materials

One hundred sixteen infertile patients were enrolled in the study. All couples underwent IVF treatment. The indication for this treatment consisted of the following: tubal factors, male factor and idiopathic infertility. Exclusion criteria for females referred to age over 39 years, FSH in the 3rd to 5th day of the cycle over 12 mIU/mL, grade III or IV endometriosis according to American Society of Reproductive Medicine (ASRM), and polycystic ovarian syndrome. For males, the exclusion criteria consisted of azoospermia and hypogonadotropic hypogonadism. The sperm parameters assessed on the day of ovum pickup were used to determine eligibility for either classical IVF or intracytoplasmic sperm injection (ICSI). Only couples with at least two mature oocytes retrieved per patient were qualified for the study. Finally, forty couples underwent conventional IVF treatment, whereas 76 couples underwent ICSI.

All the cohorts of Caucasian descent came from Central European population (Poland). Male patients with chromosomal abnormalities or erectile disorders who could not provide the sperm samples in the process of masturbation were also excluded from the study.

Patients subgrouping

The major division of patients included the method of fertilization (IVI and ICSI). Forty couples underwent conventional in vitro fertilization and 76 intracytoplasmic sperm injection. The criteria for the fertilization method choice concerned mainly sperm quality. Patients with normozoospermia (accordingly 2010 WHO manual [36]) were qualified for classical IVF, whereas in both conditions: 1) worse sperm quality, or 2) after unsuccessful IVF attempt, in patients with normozoospermia ICSI fertilization was performed. The concentration, motility, morphology, and viability of spermatozoa were significantly better in subgroups which underwent conventional IVF. Subsequently, we analyzed the polymorphisms prevalence with sperm parameters (concentration, motility, morphology, HOS [as the indicator of sperm vitality] and patients' classification to the normozoospermia group (defined using the 2010 WHO manual [36]). The polymorphisms occurring in ESR1 and *ESR2* genes were compared to the fertilization rates of oocytes fertilized in conventional IVF and ICSI as well as sperm parameters.

For every seminal parameter, we divided the patients into two groups – parameters either below or within the normal range as determined by the 2010 WHO manual [36]. Additionally, we analyzed the genotype distribution in the patient subgroups with and without normozoospermia, as confirmed by concentration, motility with progressive movement, and sperm morphology. This was performed to assess whether the analyzed genotypes are dominant in the above-mentioned subgroups.

Methods

Semen Analysis

On the day of ovarian puncture, semen samples were obtained from patients following three days of sexual abstinence. All samples underwent standard evaluation of concentration, motility, morphology, and viability according to the 2010 WHO manual. Hypoosmotic test (HOS) was used as a viability marker. Samples with leukocyte concentration >106/mL were excluded from the study.

In vitro procedure

Conventional IVF and ICSI were performed according to the standards of the Department of Infertility and Reproductive Endocrinology [10]. For ovarian stimulation, a protocol utilizing a GnRH agonist has been described previously [34]. Briefly, when the dominant follicles had a diameter > 17 mm and estradiol concentrations were 150-200 pg/mL/follicle, 10,000 IU of human chorionic gonadotropin (HCG; Pregnyl, Organon) was injected intramuscularly to facilitate final oocyte maturation. After 36 hours, the ovaries were punctured under transvaginal ultrasonography control and the follicular fluid was aspirated to obtain the oocytes. The retrieved oocytes were evaluated microscopically and only mature cells in MII were qualified for further IVF processing.

After liquefaction, the sperm samples were prepared by centrifugation using a SpermGrad (Vitrolife, Sweden). Spermatozoa were then dissolved in G-IVF Plus medium (Vitrolife, Sweden) and incubated in 37°C for two hours prior to the IVF procedure.

For conventional IVF, oocytes were placed in 5-well dishes with G-1 Plus medium (Vitrolife, Sweden), inseminated with 50.000–100.000 motile sperm, and incubated. For ICSI, MOPS Plus buffer and sperm were placed in ICSI dishes and covered with a layer of OVOIL oil (Vitrolife, Sweden). Single oocytes were placed in each well. Spermatozoa exhibiting the proper morphology and motility were immobilized and injected into the oocytes using a microinjection needle. Oocytes were then placed in G-1 Plus medium, covered with OVOIL oil, and incubated. Approximately

Table 1. Average sperm parameters in patients who underwent conventional IVF and ICSI

	IVF		**ICSI**		
	Mean ± SD	Median	Mean ± SD	Median	p
Concentration [mln/mL]	39±16	40	18±18	13	<0.0001a
Progressive motility [%]	25±9	21	14±10	12	<0.0001a
Correct morphology [%]	5±2	5	3±2	3	<0.0001a
HOS [%]	62±11	63	48±19	50	<0.0001b

IVF – patients treated with conventional in vitro fertilization, ICSI – patients treated with intracytoplasmic sperm injection; HOS – hypoosmotic test, SD – standard deviation; a – Mann-Whitney U test; b – Student's t-test

16 to 18 hours after insemination, fertilization was evaluated. The presence of two pronuclei in the oocyte was considered as completed fertilization process.

Sample Collection and Genetic Analysis

Sample collection. Blood samples were obtained from each male subject to isolate genomic DNA. Approximately 2.7 mL of peripheral blood was collected from all male patients in S-Monovette® EDTA tubes (SARSTEDT AG & Co., Numbrecht, Germany) for genetic analysis of *ESR1* (NCBI Gene ID: 2099) and *ESR2* (NCBI Gene ID: 2100) single nucleotide polymorphisms (SNPs).

Genotyping. The genomic DNA (gDNA) from each male patient was isolated from peripheral blood with the AxyPrep Blood Genomic DNA Miniprep Kit (Axygen Scientific, Inc. Union City, CA, USA) according to the manufacturer's protocol. DNA concentration and purity were determined spectrophotometrically, and 50 to 200 ng of gDNA was used in each polymerase chain reaction (PCR) and restriction analysis. Ten percent of the randomly selected samples were purified using AxyPrepPCR Clean-up Kit according to the manufacturer's protocol (Axygen). The samples were then sequenced to confirm identity in relation to the known sequences in the NCBI (National Center for Biotechnology Information) gene database.

For each *ESR1* and *ESR2* restriction site, gene specific primers were designed using Primer3 Plus Web Software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/Copyright© 2006, 2007 by Andreas Untergasser and Harm Nijveen) (primer sequences are found in Supplementary Table S1). Each reaction was carried out in a total volume of 25 µL containing the following: 50–200 ng of template gDNA, 300 nmol/L of forward and reverse genomic specific primers (Genomed, Gdansk, Poland), and 1x KAPA HiFi HotStart ReadyMix PCR Kit (KAPABiosystems, Boston, MA, USA). Each reaction was performed in duplicate. The PCR reaction was carried out in a MJ Mini™ gradient thermal cycler (Bio-Rad Laboratories, Inc. Hercules, CA, USA). The thermal profile for each amplicon and primer set are described in Supplementary Table S2. For visualization, 5 µL of PCR product was compared with the Nova 100 molecular mass marker (Novazym, Poznan, Poland) after electrophoretic separation in a 2% agarose gel (FMC BioProducts, Rockland, ME, USA) containing 1x Tris/Boric Acid/EDTA (TBE) buffer (Bio-Rad) and 500 ng/ml ethidium bromide (Sigma-Aldrich, St. Louis, MO, USA).

The remaining PCR products were purified according to the AxyPrepPCR Clean-up Kit manufacturer's protocol (Axygen), and restriction fragments length polymorphism (RFLP) analysis was conducted. The purified PCR product was used in each restriction analysis for *Xba*I, *Pvu*II, *Alu*I, *Rsa*I, and *AlwN*I. Each restriction reaction was conducted in reaction mixture containing 1.5 µL PCR product, 1x FastDigest Green Buffer, 1 FDU (fast digest unit) of the FastDigest Enzyme (Thermo Scientific, Waltham, MA, USA), and DNase free water. The total volume of the restriction reaction was 15 µL. The restriction sites and polymorphisms for each enzyme are described in Supplementary Table S3. The restriction solutions were incubated for 1 h at 37°C and FD enzyme thermal inactivation was performed for 10 min at 85°C. The RFLP reactions were electrophoresed as described above. Polymorphisms of *ESR1* and *ESR2* were identified on an agarose gel after visualizing one, two, or three bands, which corresponded to the genetic homo- or heterozygous alleles. Information included in the studies reporting genetics of infertility are summarized in Supplementary Tables S4 and S5 are according to Traven et al. [35].

Statistical analysis

Statistical analysis was performed using Statistica 13 (TIBCO Software, Tulusa, USA). Deviation from the HWE was examined using the Michael H. Court's (2005–2008) online calculator Excel-based HWE Test (https://www.tufts.edu/). The distributions of sperm laboratory parameters were assessed with the Shapiro-Wilk test, and the average sperm parameters were compared using Student's t-test or the Mann-Whitney U test. Sperm parameters and fertilization rates vs. genotype/allele distributions were assessed with the chi-squared tests according to Cochran's Rule for contingency tables. Comparisons of median sperm parameters and fertilization rate of conventional IVF and ICSI between the genotypes for *ESR1* and *ESR2* SNPs were assessed using Mann-Whitney U and Kruskal-Wallis tests. The post-hoc test with the number of participants revealed power ranged from 0.05 to 0.99. The calculation was performed with the G*Power software version 3.1.9.2 [16].

Table 2. *ESR1* and *ESR2* SNPs vs. sperm parameters

Genotype	Sperm concentration		p	Sperm progressive motility		p	Sperm morphology		p	HOS		p	Normozoospermia		p
	< 15 mln/mL	≥ 15 mln/mL		< 32%	≥ 32%		< 4%	≥ 4%		< 58%	≥ 58%		yes	no	
ESR1															
***Pvu*II**	**35**	**67**		**77**	**25**		**33**	**69**		**54**	**48**		**23**	**79**	
TT	11 (31%)	13 (19%)		19 (25%)	5 (20%)		8 (24%)	16 (23%)		12 (22%)	12 (25%)		5 (22%)	19 (24%)	
TC	16 (46%)	36 (54%)	0.4[a]	38 (49%)	14 (56%)	0.83[a]	17 (52%)	35 (51%)	0.98[b]	25 (46%)	27 (56%)	0.33[a]	13 (56%)	39 (49%)	0.82[a]
CC	8 (23%)	18 (27%)		20 (26%)	6 (24%)		8 (24%)	18 (26%)		17 (32%)	9 (19%)		5 (22%)	21 (27%)	
Power[d]	0.65			0.24			0.07			0.72			0.25		
T	38 (54%)	62 (46%)	0.28[a]	76 (49%)	24 (48%)	0.87[a]	33 (50%)	67 (49%)	0.85[a]	49 (45%)	51 (53%)	0.27[a]	23 (50%)	77 (49%)	0.85[a]
C	32 (46%)	72 (54%)		78 (51%)	26 (52%)		33 (50%)	71 (51%)		59 (55%)	45 (47%)		23 (50%)	82 (51%)	
Power[d]	0.63			0.06			0.06			0.63			0.06		
***Xba*I**	**35**	**67**		**77**	**25**		**33**	**69**		**54**	**48**		**23**	**79**	
AA	7 (20%)	11 (16%)		15 (19%)	3 (12%)		7 (21%)	11 (16%)		11 (20%)	7 (15%)		2 (9%)	16 (20%)	
AG	14 (40%)	31 (46%)	0.81[a]	35 (46%)	10 (40%)	0.46[b]	16 (49%)	29 (42%)	0.51[a]	23 (53%)	22 (46%)	0.75[a]	9 (39%)	36 (46%)	0.22[a]
GG	14 (40%)	25 (38%)		27 (35%)	12 (48%)		10 (30%)	29 (42%)		20 (27%)	19 (39%)		12 (52%)	27 (34%)	
Power[d]	0.21			0.74			0.66			0.70			0.99		
A	28 (40%)	53 (40%)	0.95[a]	65 (42%)	16 (32%)	0.2[a]	30 (46%)	51 (37%)	0.25[a]	45 (35%)	36 (38%)	0.54[a]	13 (28%)	68 (43%)	0.07[a]
G	42 (60%)	81 (60%)		89 (58%)	34 (68%)		36 (64%)	87 (63%)		63 (65%)	60 (62%)		33 (72%)	90 (57%)	
Power[d]	0.05			0.82			0.73			0.06			0.99		
ESR2															
***Alu*I**	**31**	**61**		**71**	**21**		**31**	**61**		**49**	**43**		**19**	**73**	
AA	3 (10%)	5 (8%)	1[b]	6 (8%)	2 (10%)	0.49[b]	2 (6%)	6 (10%)	0.54[b]	5 (10%)	3 (6%)	0.78[b]	2 (11%)	6 (8%)	0.21[b]
AG	13 (42%)	26 (43%)		28 (40%)	11 (52%)		11 (36%)	28 (46%)		19 (39%)	20 (47%)		11 (58%)	28 (38%)	

Table 2. *ESR1* and *ESR2* SNPs vs. sperm parameters

GG	15 (48%)	30 (49%)		37 (52%)	8 (38%)		18 (58%)	27 (44%)		25 (51%)	20 (47%)		6 (31%)	39 (54%)	
Power[d]	0.08			0.67			0.72			0.35			0.72		
A	19 (31%)	36 (30%)	0.87[a]	40 (28%)	15 (36%)	0.35[a]	15 (24%)	40 (33%)	0.23[a]	29 (30%)	26 (30%)	0.92[a]	15 (39%)	40 (27%)	0.15[a]
G	43 (69%)	86 (70%)		102 (72%)	27 (64%)		47 (76%)	82 (67%)		69 (70%)	60 (70%)		23 (61%)	106 (73%)	
Power[d]	0.06			0.68			0.82			0.05			0.92		
***Rsa*I**	**31**	**56**		**67**	**20**		**28**	**59**		**46**	**41**		**18**	**69**	
GG	26 (84%)	53 (95%)	0.13[b]	60 (90%)	19 (95%)	0.68[b]	23 (82%)	56 (95%)	0.11[b]	40 (87%)	39 (95%)	0.27[b]	17 (94%)	62 (90%)	1[b]
GA	5 (16%)	3 (5%)		7 (10%)	1 (5%)		5 (18 %)	3 (5%)		6 (13%)	2 (5%)		1 (6%)	7 (10%)	
Power[d]	0.8			0.34			0.88			0.6			0.34		
G	57 (92%)	109 (97%)	0.14[b]	127 (95%)	39 (98%)	0.68[b]	51 (91%)	115 (97%)	0.11[b]	86 (93%)	80 (98%)	0.28[b]	35 (97%)	131 (95%)	1[b]
A	5 (8%)	3 (3%)		7 (5%)	1 (2%)		5 (9%)	3 (3%)		6 (7%)	2 (2%)		1 (3%)	7 (5%)	
Power[d]	0.68			0.44			0.79			0.73			0.34		
***AlwN*I**	**36**	**60**		**74**	**22**		**33**	**63**		**53**	**43**		**20**	**76**	
AA	0	4 (7%)		2 (3%)	2 (9%)		0	4 (6%)		1 (2%)	3 (7%)		2 (10%)	2 (3%)	
AT	13 (36%)	15 (25%)	0.21[b]	23 (31%)	5 (23%)	0.36[b]	14 (42%)	14 (22%)	0.06[c]	17 (32%)	11 (26%)	0.39[b]	4 (20%)	24 (31%)	0.23[b]
TT	23 (64%)	41 (68%)		49 (66%)	15 (68%)		19 (58%)	45 (72%)		35 (66%)	29 (67%)		14 (70%)	50 (66%)	
Power[d]	0.99			0.92			0.99			0.91			0.84		
A	13 (18%)	23 (19%)	0.84[a]	25 (17%)	7 (16%)	0.94[a]	14 (21%)	22 (17%)	0.53[a]	19 (18%)	17 (20%)	0.75[a]	8 (20%)	28 (18%)	0.82[a]
T	59 (82%)	97 (81%)		121 (83%)	35 (84%)		52 (79%)	104 (83%)		87 (82%)	69 (80%)		32 (80%)	124 (82%)	
Power[d]	0.07			0.07			0.27			0.11			0.11		

SNP – single nucleotide polymorphism, HOS – hypoosmotic test; [a] – Pearson's chi-squared p-value; [b] – Fisher's exact test p-value; [c] –Fisher-Freeman-Halton exact test p-value; Power[d] – G*Power power post-hoc calculated

Table 3. Comparison of median sperm parameters and fertilization rate of conventional IVF and ICSI between the genotypes for *ESR1* and *ESR2* SNPs

Genotype	Sperm concentration [mln/mL]	Sperm motility [%]	Sperm morphology [%]	HOS [%]	Oocytes fertilized in conventional IVF [%]	Oocytes fertilized in ICSI [%]
			ESR1			
PvuII TT TC CC	p = 0.36[a]	p = 0.6[a]	p = 0.9[a]	p = 0.54[a]	p = 0.16[a]	p = 0.75[a]
XbaI AA AG GG	p = 0.31[a]	p = 0.71[a]	p = 0.33[a]	p = 0.78[a]	p = 0.73[a]	p = 0.97[a]
			ESR2			
AluI AA AG GG	p = 0.94[a]	p = 0.88[a]	p = 0.69[a]	p = 0.72[a]	p = 0.25[a]	p = 0.97[a]
RsaI GG GA	p = 0.32[b]	p = 0.71[b]	p = 0.21[b]	p = 0.47[b]	p = 0.53[b]	p = 0.67[b]
AlwNI CC CT TT	p = 0.1[a]	p = 0.12[a]	p = 0.31[a]	p = 0.4[a]	p = 0.79[a]	p = 0.86[a]

SNP – single nucleotide polymorphism, ***ESR1***– estrogen receptor 1 gene, ***ESR2*** – estrogen receptor 2 gene, HOS – hypoosmotic test, IVF – in vitro fertilization, ICSI – intracytoplasmic sperm injection; [a] – Kruskal-Wallis test, [b] –Mann-Whitney U test

RESULTS

The average age of male patients was 34 years, and their partners mean age was 32. The mean seminal parameters of the entire study group were as follows: sperm concentration of 2.5E7/mL, 18% sperm showing progressive motility, 4% spermatozoa with normal morphology and 53% live sperm. As defined using the 2010 WHO manual, normozoospermia, in all basic parameters (motility, concentration, and morphology), was observed in 28 patients (24% of the study group) [36]. Sperm parameters in the subgroups which underwent conventional IVF and ICSI are presented in Table 1. The concentration, motility, morphology, and viability of spermatozoa were significantly better in the subgroups which underwent conventional IVF. There was no difference in the average fertilization rate between groups (67% vs. 73%; p>0.05).

Restriction analysis was fully conclusive for 102 patients in the case of *ESR1* and 92 in the case of *ESR2*. Despite the fact that the patients were a selected population treated by in vitro fertilization, all of evaluated SNPs distributions were consistent with the Hardy-Weinberg equilibrium (HWE). *ESR1* and *ESR2* genotypes did not differ significantly from those expected under HWE (p>0.05 for all genotypes), although for *Alu*I analysis, only eight patients had genotype AA. For *Rsa*I, genotype AA was not observed in any patient, and genotype GA was seen in eight patients. Concerning *AlwN*I restriction site, genotype TT was present just in four males. Absence of AA genotype analyzed by *RsaI* is consistent with the literature data as this genotype was marked the least frequent, regardless of the studied male population [19, 24, 37]. Concerning the distribution of genotypes, it is worth noting this study included a selected group of men, most of whom displayed decreased sperm parameters.

Using the chi-squared test and the Fisher exact test, we evaluated the association between sperm parameters and *ESR1* and *ESR2* polymorphisms. For *ESR1*, no predominance of any of the analyzed genotypes was found for any of the sperm parameters. The lowest p-value obtained was 0.07, and thus no statistical significance was found. This means that in the study population, the correct semen parameters, either oligozoospermia, asthenozoospermia or teratozoospermia, were not related to rs2234693 and rs9340799 polymorphisms. No relationship was observed between the studied seminological parameters and the genotypes of the *ESR2* restriction sites. Only in the case ofrs1256120, the TT genotype is predominant among patients with ≥4% normal sperm morphology; however, this result is not statistically significant (p = 0.06) (Table 2).

Similarly, we observed no differences in sperm parameters between the genotypes using the Kruskal-Wallis and Mann Whitney U tests (p>0.05 for all sperm parameters in every SNP) (Table 3). For *ESR1* polymorphisms, as related to sperm motility, rs2234693 and rs9340799 did not differ significantly between genotypes. Analysis of *Alu*I, *Rsa*I, and *AlwN*I restriction sites, all associated with

the *ESR2* gene, no correlation of genotypes was observed with sperm parameters. In the *Alu*I study, p-values of 0.94, 0.69, and 0.88 (sperm concentration, morphology, and motility, respectively) between individual genotypes indicated no differences in analyzed parameters depending on the genotype. The results of sperm parameters indicate lower p-values within the rs1256120 TT genotype. However, there were no statistical differences observed ($p = 0.1$, $p = 0.12$, and $p = 0.31$ for concentration, motility, and morphology, respectively). In the case of *RsaI* restriction analysis, improved sperm parameters for the GG genotype were observed, but no statistical difference was found for any parameter ($p = 0.32$, $p = 0.71$, and $p = 0.21$ for concentration, motility, and morphology, respectively). In the studied population, however, no relationship between *ESR2* polymorphisms and the seminological parameters has been shown (Table 3). Within the *Pvu*II restriction site, there was no difference between the effectiveness of in vitro fertilization (measured by percentage of fertilized oocytes), regardless of genotype, both in the case of conventional IVF or ICSI ($p = 0.16$ and $p = 0.75$, respectively). For the *Xba*I restriction site, studies comparing the efficacy of conventional IVF and ICSI showed no difference. The association of XbaI restriction site polymorphisms with the effectiveness of fertilization by any of the methods used in *in vitro* fertilization may be excluded (IVF $p = 0.73$, ICSI $p = 0.97$). Due to the *ESR2* localization in the midpiece area of the sperm, as well as the role of estrogens in the fertilization process, we analyzed the relationship of *ESR2* polymorphisms with conventional IVF and ICSI fertilization rate no statistically significant relationship between individual genotypes with fertilization effectiveness in any of the methods. In the case of conventional IVF, we can observe a tendency towards a slightly lower percentage of fertilized egg cells for the AA genotype ($p = 0.25$; rs4986938; 1730 G>A) (Table 3).

Furthermore, we analyzed the association between SNP genotypes and fertilization rates from two methods of *in vitro* fertilization. We divided the patients into conventional IVF and ICSI groups. The patients were then further subdivided by fertilization of at least one oocyte or no oocytes. We used the chi-squared test to analyze the genotype distribution according to fertilization success. In the group which underwent conventional IVF, the males who were heterozygote TC carriers for the SNP recognized by *Pvu*II had an improved treatment outcome than patients who did not have this genotype ($p = 0.02$). This was not observed for any of the *ESR1* and *ESR2* SNPs (Table 4).

For *Alu*I and *AlwN*I restriction sites, there were no differences between genotypes and fertilization success in any of the utilized methods. Rs1256049 polymorphism (recognized by *Rsa*I) and IVF efficacy, due to lack of AA genotype and low GA group genotype, were analyzed using the chi-squared test. This was performed to compare patients who had fertilized at least one oocyte to those with none. No relationship was found from this comparison in either the IVF or the ICSI groups (Table 4).

DISCUSSION

To assess the relationship of sperm parameters with the *ESR1* and *ESR2* gene polymorphisms, we proposed a comparison of their mean values within genotypes to the examined restriction sites. The presence of receptors in the sperm mitochondria, depending on the gene variant, could be related to varying mobility. However, the results did not confirm a correlation. The lack of association of different genotypes and semen parameters may be related to a relatively small population of normozoospermia cases. Normozoospermia was seen in 23 patients subjected to the analyses using *Pvu*II (rs2234693; 397 T>C) and *Xba*I (rs9340799; 351 A>G), 19 for *Alu*I (rs4986938; 1730 G>A), and 18 for *Rsa*I (rs1256049; 1082 G>A). In the case of *AlwN*I (rs1256120; -458 T>C), normozoospermia existed in 20 individuals. This may explain the differing observations from those presented by the Lazaros et al. [24]. These authors found the relationship of the rs1256120 (defined by *AlwN*I) to higher gamete mobility as compared to the AG and AA genotypes; however, in the studied population of 114 men, as many as 85 had normozoospermia. However, it cannot be ruled out that, due to the post-genomic effect of estrogen receptors on sperm, structural disturbances within the restriction sites are not related to mitochondrial function and gamete mobility.

We found no statistically significant differences for the *ESR1* and *ESR2* restriction sites, indicating none of the genotypes were associated with the occurrence of normozoospermia in patients. The results presented are partly consistent with those published by Lazaros et al. [24]. Their work analyzed genotype distribution in patient groups with normal concentration and sperm motility then assessed groups with oligozoospermia and asthenozoospermia. They found that rs2234693 and rs9340799 polymorphisms did not correlate with sperm motility in patients with normal sperm kinetics. They noticed, however, in the asthenozoospermia group, CC and TC genotypes were associated with improved gamete motility than was the cytosine-free genotype, i.e. TT. In the case of *Xba*I restriction analysis, better sperm motility was demonstrated in patients with genotypes AG and GG than in the case of AA [23].

In the analysis of sperm concentration as related to rs2234693 and rs9340799 polymorphisms (defined by *Pvu*II and *XbaI,* respectively), Lazaros et al. noted that the genotype TT analyzed by *Pvu*II is accompanied by a larger amount of sperm in the group with normal sperm concentrations. For *Xba*I restriction analyses, a higher semen concentration was observed in the AA genotype as opposed to AG and GG. Among the patients with asthenozoospermia, these relationships were not observed. Concerning *ESR2,* the researchers found, contrary to the results presented here, higher values of sperm motility in progressive movement in patients with the rs4986938 GG genotype. At the same time, similar to the present study, no relationship was observed between sperm concentration and *Alu*I restriction site, nor was one noted between sperm parameters and rs1256049 genotypes. Discrepancies in these

Table 4. *ESR1* and *ESR2* SNPs vs. fertilization success in conventional IVF and ICSI

Genotype	Conventional IVF	Fertilization completed	No fertilization	p	ICSI	Fertilization completed	No fertilization	p
				ESR1				
PvuII	**36**	**29**	**7**		**66**	**61**	**5**	
TT	6 (17%)	6 (21%)	0		18 (27%)	16 (26%)	2 (40%)	
TC	20 (55%)	18 (62%)	2 (29%)	0.02c	32 (48%)	30 (50%)	2 (40%)	0.84[a]
CC	10 (28%)	5 (17%)	5 (71%)		16 (24%)	15 (24%)	1 (20%)	
Power[d]			0.99			0.64		
T	32 (44%)	30 (52%)	2 (36%)	0.02b	68 (52%)	62 (51%)	6 (60%)	0.75[b]
C	40 (56%)	28 (48%)	12 (64%)		64 (48%)	60 (49%)	4 (40%)	
Power[d]			0.77			0.06		
XbaI	**36**	**29**	**7**		**66**	**61**	**5**	
AA	4 (12%)	3 (10%)	1 (14%)		14 (21%)	13 (21%)	1 (20%)	
AG	16 (44%)	14 (49%)	2 (29%)	0.61a	29 (44%)	27 (45%)	2 (40%)	0.16[a]
GG	16 (44%)	12 (41%)	4 (57%)		23 (35%)	21 (34%)	2 (40%)	
Power[d]			0.57			0.14		
A	24 (33%)	20 (34%)	4 (29%)	0.76b	57 (43%)	53 (43%)	4 (40%)	1[b]
G	48 (67%)	38 (66%)	10 (71%)		75 (57%)	69 (57%)	6 (60%)	
Power[d]			0.14			0.06		
				ESR2				
AluI	**31**	**24**	**7**		**61**	**56**	**5**	
AA	2 (6%)	1 (4%)	1 (14%)		6 (10%)	5 (9%)	1 (20%)	
AG	14 (45%)	11 (46%)	3 (43%)	0.64a	25 (41%)	23 (41%)	2 (40%)	0.63[a]
GG	15 (49%)	12 (50%)	3 (43%)		30 (49%)	28 (50%)	2 (40%)	
Power[d]		0.72			0.79			
A	18 (29%)	13 (27%)	5 (36%)	0.52b	37 (30%)	33 (29%)	4 (40%)	0.48[b]
G	44 (71%)	35 (73%)	9 (64%)		85 (70%)	79 (71%)	6 (60%)	
Power[d]			0.36			0.76		
RsaI	**30**	**29**	**1**		**57**	**50**	**7**	
GG	24 (80%)	23 (79%)	1 (100%)	-	53 (93%)	46 (92%)	7 (100%)	-
GA	6 (20%)	6 (21%)	0		4 (7%)	4 (8%)	0	
Power[d]		-			-			
G	54 (90%)	52 (90%)	2 (100%)	-	110 (96%)	96 (96%)	14 (100%)	-
A	6 (10%)	6 (10%)	0		4 (4%)	4 (4%)	0	
Power[d]		-			-			
AlwNI	**30**	**24**	**6**		**66**	**61**	**5**	
CC	2 (13%)	2 (8%)	0		2 (3%)	2 (3%)	0	
CT	5 (17%)	4 (17%)	1 (17%)	1c	23 (35%)	23 (38%)	0	0.28[c]
TT	23 (70%)	18 (75%)	5 (83%)		41 (62%)	36 (59%)	5 (100%)	
Power[d]			0.29			0.99		
C	9 (15%)	8 (17%)	1 (8%)	0.67b	27 (20%)	27 (22%)	0	-
T	51 (85%)	40 (83%)	11 (92%)		105 (80%)	95 (78%)	10 (100%)	
Power[d]			0.46		-			

SNP – single nucleotide polymorphism, IVF – in vitro fertilization, ICSI – intracytoplasmic sperm injection; a – Pearson's chi-squared p-value; b – Fisher's exact test p-value; c – Fisher-Freeman-Halton exact test p-value; Powerd – G*Power power post-hoc calculated

results are quite difficult to interpret. The size of the study groups was comparable [24]. The authors referred to WHO standards in 1999, where the diagnosis of oligozoospermia was made when the concentration of sperm was <20 million/mL, and the motility of the gametes with progressive movement was estimated at <50% for asthenozoospermia. Additionally, the result discrepancies may be due to the different ethnic backgrounds of the groups. The origin of patients in the study group is an important factor that may affect polymorphism distribution.

Solakidi et al. reported ESR1 localized to the equatorial segment of the sperm head. They suggest the possible involvement of this receptor in combining the cell membranes of the male and female gametes [32]. It seems, therefore, that individual polymorphisms of the gene encoding *ESR1* in men could be associated with differing percentages of fertilized egg cells in their partners. For this reason, data analysis was performed in the current study. Although no difference was shown between the effectiveness of classical *in vitro* fertilization and genotype in the *Pvu*II analyses, the potency of this method seems to be slightly lower in the CC genotype than in TT and TC variants. Similar relationships were not observed in the case of ICSI. For the *Xba*I restriction site, the efficacy of conventional IVF and ICSI showed no differences, which was also observed for *Alu*I. In the case of conventional IVF, one can observe a tendency towards a slightly lower percentage of fertilized egg cells for the AA genotype. These results do not coincide with the results of Aschim et al. [2]. These researchers noticed the rarest occurrence of the AA genotype in infertile men, although no significant differences between genotypes were observed. However, this work used a different criterion for male infertility (sperm concentration <5 million/mL and no partner study), so the results are difficult to compare. For patients treated with ICSI in our work, the dominant trend of one of the genotypes analyzed by *Alu*I in the context of the effectiveness of ICSI was not observed. It can be presumed that, while ESR1 and ESR2 polymorphisms may be related to conventional IVF efficacy (and perhaps a natural conception) and in ICSI conditions, they do not affect its result.

In the *AlwN*I and *Rsa*I assessment, there were no differences between genotypes and the effectiveness of fertilization in any of the methods assessed. Zhang et al. conducted a study of this polymorphism in the context of fertilization effectiveness in the Chinese population [37]. These authors observed the occurrence of the AA genotype in 32 of 865 couples and showed that, although there was no difference between the genotypes and effectiveness of fertilization, it was the AA genotype that was associated with poorer embryo quality. These results encourage similar research in Caucasian populations.

Significantly higher occurrences of the TC genotype of the *Pvu*II restriction site were seen where at least one oocyte was fertilized ($p = 0.02$). This was not observed for other genotypes analyzed with *Pvu*II, nor for the genotypes identified by *Xba*I or any of the SNP *ESR2* genotypes. Therefore, it is the only statistically significant relationship between the studied estrogen receptor polymorphisms and fertilization effectiveness assessed in this work. Therefore, the conclusion regarding a favorable relationship between the rs2234693 TC genotype and the fertilizing capacity of semen should be treated with caution. With the present size of the group, the statistical power ranged from 0.05 to 0.99 and the continuation of research with an increase in the size of the group would be advisable, because major determinant of statistical power is allele frequency.

Because of the polygenic nature of spermatogenic disorders, additional loci could be involved in spermatozoa defects and conception ability. This may be related to estrogens, their receptors, or other core genes involved in estrogenic and estrogen-related pathways. In most cases, we did not discover a relationship between both estrogen receptors polymorphisms and sperm function, except forrs2234693 and fertilization rate. There was no association with *ESRs* polymorphisms and standard WHO semen parameters.

ETHICAL APPROVAL

All human procedural studies were conducted in accordance with the ethical standards of the institutional and /or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the patients were informed about the purpose of the study and provided written consent. The study protocol was approved by the Institutional Review Board of the Poznan University of Medical Sciences.

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The authors have no potential conflicts of interest to declare.

Table S1. ESR1 and ESR2 gene primers for PCR

Primer's name	Sequence 5' 3'	Annealing temperature	Amplicon length
ESR1_rs9340799_XbaI_F ESR1_rs2234693_PvuII_F	CTGCCACCCTATCTGTATCTTTTCCTATTCTCC	71ºC	1374 bp
ESR1_rs9340799_XbaI_R ESR1_rs2234693_PvuII_R	TCTTTCTCTGCCACCCTGGCGTCGATTATCTGA		
ESR2_ rs4986938_AluI_F	GTGTGTGGTGGGACACAGAG	65ºC	646 bp
ESR2_ rs4986938_AluI_R	AGGCCATTGAGTGTGGAAAC		
ESR2_rs1256049_RsaI_F	TTCTGAGCCGAGGTCGTAGT	66ºC	582 bp
ESR2_ rs1256049_RsaI_R	TGAATCCTTGGACCCAACTC		
ESR2_rs1256120_AlwNI_F	GACTTTGTCACACACCTGCG	68ºC	620 bp
ESR2_rs1256120_AlwNI_R	AAACAGGCCACCGTCAGAAA		

F – gene specific forward primer, R – gene specific reverse primer

Table S2. Thermal profiles for PCR reactions

Restriction site	XbaI PvuII	AluI	RsaI	AlwNI	Cycles no.
Initial denaturation	95ºC, 5 min	95ºC, 5 min	95ºC, 5 min	95ºC, 5 min	1
Denaturation	95ºC, 20 s	98ºC, 20 s	98ºC, 20 s	98ºC, 20 s	30
Annealing	71ºC, 15 s	65ºC, 15 s	66ºC, 15 s	68ºC, 15 s	
Elongation	72ºC, 15 s	72ºC, 15 s	72ºC, 15 s	72ºC, 15 s	
Final elongation	72ºC, 3 min	72ºC, 1 min	72ºC, 1 min	72ºC, 1 min	1

Table S3. Restriction enzymes and allelic sites for ESR1 and ESR2 genes

Enzyme	Restriction site	Allele	Product length
ESR1_rs9340799_XbaI	T*CTAGA	T*CTAGA	981bp + 393bp
		TCTGGA	1374 bp
ESR1_rs2234693_PvuII	CAG*CTG	CAG*CTG	936bp + 438 bp
		CAGCCG	1374 bp
ESR2_rs4986938_AluI	AG*CT	AG*CT	445 bp + 201 bp
		GGCT	646 bp
ESR2_ rs1256049_RsaI	GT*AC	GT*AC	293 bp + 289 bp
		GTGC	582 bp
ESR2_rs1256120_AlwNI	CAGNNN*CTG	CAGNNN*CTG	158 bp + 462 bp
		CAGNNNCCG	620 bp

* restriction site

Table S4. Minimum checklist of essential information included in the studies reporting genetics of infertility, source databases and examples

Information		Input	Source database
Locus information	Locus biotype/ sequence feature/ sequence variant	Single nucleotide polymorphism (SNP)	The Sequence ontology (http://www.sequenceontology.org/)
	Sequence ontology accession	SO:0001969, SO:0002153, SO:0001580, SO:0001624	The Sequence ontology (http://www.sequenceontology.org/)
	Locus name / gene symbol	*ESR1*, *ESR2*	HGNC (http://www.genenames.org/)
	Gene name	Estrogen receptor 1 Estrogen receptor 2	HGNC (http://www.genenames.org/)
	Entrez Gene ID	2099 (*ESR1*); 2100 (*ESR2*)	Entrez (www.ncbi.nlm.nih.gov)
	Chromosome number	6 (*ESR1*); 14 (*ESR2*)	Ensembl (http://www.ensembl.org)
	Genomic coordinate of the polymorphism, locus	Chromosome # 6q25.1-q25.2 (ESR1) 151656691-152,129619 (protein coding gene) (ESR1) 151842246-151842246 (SNP) 151842200-151842200 (SNP) Chromosome # 14q23.2-q23.3 (ESR2) 64084232-64338112 (protein coding gene) (ESR2) 64338283-64338283 (SNP) 64257333-64257333 (SNP) 64233098- 64233098 (SNP)	dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) (SNP) Ensembl (http://www.ensembl.org) (gene)
Subjects	Race / ethnicity	Caucasian, Polish	Research
	Number of participants (infertile/controls) - include sex in each group I(M/F) C(M/F)	Total study population N= 116 Group 1= 40, Group 2 = 76 Only males in each group	Research
	Methodology	PCR followed by restriction length fragment polymorphism (PCR-RFLP)	Research
Phenotype information	Clinical data	Male patients: exclusion criteria: azoospermia, hypogonadotropic hypogonadism; erectile disorders. inclusion criteria: sperm samples provided in the process of masturbation. IVF: oocytes fertilized in standard in vitro fertilization method ICSI: oocytes fertilized by intracytoplasmic sperm injection Inclusion criteria: obtaining at least two mature oocytes from ovarian puncture Exclusion criteria were as follows: over 39 years of age, FSH >12 mIU/mL. PCS, endometriosis (grade 3 or 4).	Research
	Disease ontology	5223	Disease Ontology (http://disease-ontology.org/)
	Disease comorbidity	Not available	Research
Reference	PubMed ID (in review papers)	No review paper referenced in the paper	Research
	Reference (in review papers)	No review paper referenced in the paper	Research

Table S5. Additional information in studies reporting genetics of infertility. The format depends on locus biotype and study approach

Information	Input	Source
Reference SNP ID number (rs#)	*ESR1* rs2234693, *ESR1* rs9340799, *ESR2* rs1256120, *ESR2* rs1256049, *ESR2* rs4986938	dbSNP (http://www.ncbi.nlm.nih.gov/SNP/)
Polymorphism biotype	rs2234693, rs9340799 – intron variant rs1256120 – Genic Upstream Transcript Variant rs1256049 – Synonymous Variant rs4986938 – 3 Prime UTR Variant	dbSNP (http://www.ncbi.nlm.nih.gov/SNP/)
Minor allele frequency (MAF)	rs2234693 C = 0.46781/125568, TOPMED rs9340799 G = 0.31393/125568, TOPMED rs1256120 G = 0.21146/125568, TOPMED rs1256049 T = 0.06595/246214, GnomAD rs4986938 T = 0.31057/239640, GnomAD	dbSNP (http://www.ncbi.nlm.nih.gov/SNP/)
P value	0.02	Research
Odds ratio (OR)	n/a	Research
Method / platform - details	PCR-RFLP	Research
Repeat unit	Not applicable	Research
Number of repeats	Not applicable	Research
Gene regulation	Not applicable	Research
MicroRNA (miRBase ID)	Not applicable	Sanger miRBase (http://www.mirbase.org/)
Disease comorbidity, associated syndrome	Not applicable	Research
CNV type	Not applicable	Research
Overlapping gene	Not applicable	Research
Epigenetic mechanism	Not applicable	Research
Chromosomal aberration	Not applicable	Research