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The Impact of Genetics Profile (Gene Polymorphisms) in Obese Non-Pcos Women Entering an IVF/ICSI Program

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Abstract: Data concerning the effects of increased body mass index (BMI) on ovarian and pregnancy outcome are rich, but the results are rather controversial. Regarding pharmacogenetics, gene polymorphisms of hormonal receptor genes, such as Estrogen Receptor alpha (ESR1), Estrogen Receptor beta (ESR2) and FSH receptor (FSHR) genes, are associated with ovarian stimulation and pregnancy outcome and may constitute a useful tool for ART experts for the prediction of this outcome. The aim of this study is to track differences in the distribution of gene polymorphisms among obese non-PCOS and non-obese patients concerning three distinct genes which are involved in the ovarian stimulation mechanism: PvuII polymorphism of ESR1 gene, RsaI polymorphism of ESR2 gene and Ser680Asn variation of FSHR gene, using restriction fragment length polymorphism analysis and real-time polymerase chain reaction. A total of 151 normally ovulating female patients underwent IVF or ICSI. Interestingly, the pregnancy rate in the BMI≥30 kg/m² group was higher in a statistically significant way (40.9% versus 17.8%, p=0.023). The obese patients of this study were in need of increased total FSH dose in order to achieve a satisfactory oocyte number (p<0.001) and needed more days of stimulation (p=0.002), but also presented lower basal FSH levels (p=0.032), which may explain, to an extend, the better pregnancy outcome. Concerning the polymorphisms of ESR1, ESR2 and FSHR genes, we did not observe differences in the genotype distribution when we compared the obese non-PCOS population with the non-obese population. Thus, obesity does not constitute an additional indication to perform a genetic analysis before entering an IVF/ICSI program.

Keywords: ESR1 gene, ERS2 gene, FSH receptor gene, obesity, IVF/ICSI, polymorphisms, single nucleotide.

BACKGROUND

Obesity is a major health problem, which concerns numerous medical specializations, because it is associated with many adverse health consequences. Concerning the field of reproduction, significant associations are seen between excess body fat, especially abdominal obesity, and irregular menstrual cycles, reduced spontaneous and induced infertility, increased risk of miscarriages and hormone-sensitive carcinomas [1-4]. Polycystic ovary syndrome (PCOS) is the most common ovulation disorder associated with obesity, but it seems that obesity also adversely affects the endometrium, implantation and early fetal development [5-7].

The studies that involve anthropometric indicators and response to gonadotrophins for ovulation induction in in-vitro-fertilization (IVF) programs began in the mid nineties [8]. So far, data concerning the effects of increased body mass index (BMI) on ovarian and pregnancy outcome are rich. The results are rather controversial: there are investigators that underline an impaired ovarian response in obese patients, but comparable pregnancy rates among normal and obese patients [9, 10], while others note poor implantation, pregnancy and live birth rates as BMI increases, suggesting an alteration in the uterine environment, without differences in embryo quality [11, 12]. Some research teams noted that increased BMI is correlated with higher cancellation rates [10, 13]. Finally, there are investigators who support that excess weight alters all parameters, leading to lower stimulation response and lower live birth rates [14, 15] while others find no difference in ovarian and pregnancy outcome between obese and non-obese patients [16, 17].

Regarding pharmacogenetics, gene polymorphisms of hormonal receptor genes, such as ESR1, ESR2 and FSH receptor (FSHR) genes, are associated with ovarian stimulation and pregnancy outcome in several studies and may constitute a useful tool for ART experts for the prediction of this outcome [18-24]. Poor responders is a group of patients that seem to qualify for a genetic control prior to IVF or ICSI, as well as patients that present with many failures in previous IVF/ICSI attempts [25, 26]. Although the association of BMI with IVF parameters is a common, and rather controversial, field for research, there is still no evidence about a possible association of genetic markers, such as hormonal receptor gene polymorphisms, with BMI. Such polymorphisms have been investigated only concerning the PCOS patients [27, 28], but never in an obese non-PCOS population entering IVF programs. Until now, there has been no evidence that these polymorphisms are associated with ovarian and pregnancy outcome in this special group.

The aim of this study is to focus on tracking differences in the distribution of gene polymorphisms among obese non-PCOS and non-obese patients concerning three distinct genes which are involved in the ovarian stimulation mechanism:

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Pvu II polymorphism of ESR1 gene, Rsa I polymorphism of ESR2 gene and Ser680Asn variation of FSHR gene polymorphisms, all associated with a poor response to ovarian stimulation [26, 29], in order to establish whether a genetic investigation prior to IVF/ICSI would be helpful in planning a therapeutic strategy in this group.

METHODS AND PROCEDURES

Patients

A total of 151 normally ovulating female patients (mean age 35±5 years, mean±SD) who underwent IVF or ICSI at the IVF Unit of the "Alexandra" Maternity Hospital of the 1st Obstetrics and Gynecology Department of Athens University Medical School, Greece participated in this retrospective study. Group A consisted of 129 patients with BMI <30 kg/m². Group B consisted of 22 patients with BMI ≥30 kg/m². Institutional review board approval was obtained. All patients had at least one year of infertility before entering the study. Other inclusion criteria were a regular menstrual cycle of 25 to 35 days, age ≤45 years old and the presence of both ovaries. Patients with polycystic ovarian syndrome (as described by Rotterdam criteria) or other endocrine diseases were excluded from the study.

For 99 patients, this was their first IVF/ICSI treatment cycle, 33 patients had one previous unsuccessful cycle, and 19 patients had already two or more unsuccessful cycles. Their main indications for IVF/ICSI were as follows: tubal factor infertility (47.7%, *n=72*), male factor infertility (46.4%, *n=70*), endometriosis (1.32%, *n=2*) and unexplained infertility (1.32%, *n=2*). Few of these women had a double etiology for infertility (3.3%, *n=5*). 52% of the patients underwent ICSI, 48% underwent IVF. There were two patients that produced 2 follicles after controlled ovarian stimulation (COS), but no oocytes were retrieved afterwards (cycle cancellation).

Hormone Assays

Basal (day 3) serum FSH, LH and prolactin levels were measured by electrochemiluminescence immunoassay (Roche Molecular, Biochemicals, Mannheim, Germany) in the cycle just before the ovarian stimulation. The oestradiol (E2) level was measured on the 5th day of the COS and every day until the day of hCG administration, using a commercially available chemiluminescent Microparticle Immunoassay (CMIA) kit (Abbott Laboratories, Abbott Park, IL, USA). Prolactin levels were measured for a better evaluation of the patients, since unusually high serum prolactin levels could lead to cycle cancellation and in these cases appropriate treatment prior to initiation of an IVF cycle was given.

Ovarian Stimulation

COS was conducted according to the GnRH agonist protocol, as described previously [23, 30]. Briefly, patients <35 years old were stimulated by a long GnRH agonist protocol: on day 21 of the previous cycle, a baseline ultrasound scan was performed and buserelin acetate intranasal spray administration began at a dose of 100 μg five times per day. GnRH agonist administration was maintained until hCG administration. The extent of ovarian suppression was evaluated by ultrasound scan and serum E2 levels (≤40 pg/mL) before starting exogenous gonadotrophin administration (about 15 days after administering the spray). After a follow up of the gonadotropin administration, hCG was given when at least two follicles were larger than 18 mm and serum oestrogen levels were rising.

Oocytes were retrieved 36 h after the administration of 10.000 IU hCG. Follicular aspiration and oocyte retrieval were performed by transvaginal ultrasound guided puncture. ICSI was performed when needed, only in mature oocytes which had extruded the first polar body (metaphase II) [31].

Patients ≥35 years old were stimulated by a short GnRH agonist protocol with buserelin (500 μg/day intranasal) on cycle day 2. Gonadotrophin administration began on day 3 at a dose of 200 IU of rFSH.

Plasma E2 levels were measured daily starting 5 days after commencing the regimen until the day after hCG administration. The first scan was performed on day 7 and subsequent scans were performed every day until oocyte retrieval.

The dose of rFSH was adjusted according to ovarian response 6 days after the onset of gonadotrophin administration. GnRHa administration was continued until 10.000 IU of human chorionic gonadotropin (hCG) were injected intramuscularly. hCG was administered when the mean diameter of at least two leading follicles was >18 mm and serum E2 level was rising.

All the protocols used in these groups of patients have been previously described in detail [32]. All ultrasound scans were performed by the same clinician. A single experienced clinician performed all embryo transfers.

Embryos were scored and chosen for transfer based on rapid cleavage, absence of fragmentation, and size of blastomeres (good quality, A; poor quality, B). Pregnancy was defined as a positive biochemical pregnancy test 18 days after oocyte retrieval. Clinical pregnancy was defined if a gestational sac was present on ultrasound at six gestational weeks.

Single Nucleotide Polymorphism Genotyping

Genomic DNA was obtained from peripheral blood leucocytes with the QIAamp DNA Blood Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Patients were genotyped for PvuII (T/C, rs2234693) polymorphism in *ESR1* intron 1, RsaI (G/A, rs 1256049) polymorphism in *ESR2* exon 5, as well as for the sequence of the polymorphism of FSH receptor identified as Ser680Asn, using restriction fragment length polymorphism (RFLP) analysis-classical PCR method and real-time PCR (RT-PCR). After the DNA extraction and before practicing all PCR methods, the DNA concentration of each sample was controlled with photometry (Qubit TM Fluorometer of Invitrogen). Genotyping of Ser680Asn polymorphism of FSH receptor was performed as described in Loutradis *et al*, 2006 [23]. Genotyping of PvuII (T/C) polymorphism of ESR1 gene and RsaI (G/A) polymorphism of ESR2 gene was performed with real-time PCR, using the Light Cycler 480 II (Roche Diagnostics, Germany). The conditions of the real-time PCR for the ESR1 and ESR2 polymorphisms are described elsewhere [26].

Statistical Analysis

Statistical analysis was performed with Statistics Package for Social Sciences (SPSS), version 15, Minitab 12, while the Sasieni algorithm (1997) and Hardy-Weinberg equilibrium were performed with the on line calculator which is available on http://ihg.gsf.de. For the statistical analysis we initially investigated the normality of the analyzed variables since most gynecological parameters deviate from normality. We tested the difference observed between women above or below BMI index 30 kg/m² either by the t-test or the Mann-Whitney test according to the distribution of the parameter under investigation. The association between pregnancy outcome and BMI status was assessed by the chi-square test.

A p-value less than 0.05 was regarded as statistically significant. Values are presented as mean±SD, unless otherwise stated.

RESULTS

Patients' Characteristics and Biological and Clinical Outcome of the IVF/ICSI Cycles

The biological and clinical parameters according to BMI are shown in Table **1**. The mean age of the patients in group A (BMI<30 kg/m²) was 34.7±4.9 (±SD) years old and in group B (BMI≥30 kg/m²) was 33.9±6.1 (±SD) years old (NS). The mean BMI of the patients in group A was 23.1±2.8 kg/m², while in group B the mean BMI was 34.3±4.3 kg/m². The basal serum FSH in group A and group B was 7.7±2.9 mIU/L and 6.3±1.9 mIU/L, respectively (p=0.032). The basal serum LH in group A and group B was 5.5±2.5 mIU/L and 4.7±2.1 mIU/L, respectively (NS).

As far as the ovarian stimulation parameters and pregnancy rates are concerned, the group of women with BMI≥30 kg/m² differed in a statistically significant way from the group of women with BMI<30 kg/m² in the total FSH dose used and in the days of stimulation (Table **2**). Women with increased BMI were in need of increased total FSH dose in order to achieve a satisfactory oocyte number (p<0.001) and needed more days of stimulation (p=0.002). There were no differences in the rest of ovulation induction parameters. Comparing the groups of patients with BMI<30 kg/m² and BMI≥30 kg/m², pregnancy rate in the second group was higher in a statistically significant way (40.9% versus 17.8%, p=0.023).

Associations of Gene Polymorphisms with BMI

The distribution of *ESR1 PvuII* genotypes among the total 151 patients was as follows: 25.2% (38/151) were homozygous for TT, 53.6% (81/151) were TC and 21.2% (32/151) were homozygous for CC, with T and C allele frequencies 52 and 48%, respectively. The *ESR2 RsaI* genotypes were distributed as follows: 93.4% (141/151) were GG, 6.0% (9/151) were GA and one woman (0.6%, 1/151) had AA genotype, which gave the G and A allele frequencies an incidence of 96.4 and 3.6%, respectively. The prevalence of genotypes of the *Ser680Asn* polymorphism of the FSH receptor was: Ser/Ser 20.5% (31/151), Ser/Asn 51.7% (78/151) and Asn/Asn 27.8% (42/151), with Ser and Asn allele frequencies 46.4 and 53.6%, respectively. Genotype frequencies observed during this study are in accordance with the Hardy-Weinberg equilibrium law.

The distribution of the different genotypes of the three polymorphisms in patients with BMI<30 kg/m² and BMI≥30 kg/m² is shown in Table **3**. No significant difference was found in the genotype distribution among obese and non obese patients concerning the polymorphisms of ESR1, ESR2 and FSHR genes, although the obese patients present a higher proportion of CC genotype of ESR1 gene compared with non obese women and a lower proportion of Ser/Ser genotype of FSHR gene compared with non-obese patients. Regarding the penetrance of obesity in each genotype for the ESR1 gene, 6/38 (15.8%) TT patients, 9/81 (11.1%) TC patients and 7/32 (21.9%) CC patients were obese, a difference without statistical significance. Similarly, for the FSHR gene, 3/31 (9.7%) Ser/Ser patients, 12/78 (15.4%) Ser/Asn patients and 7/42 (16.7%) Asn/Asn patients were obese, without reaching a statistical significance.

Table 1. Descriptive Statistics of Basic Demographic and Clinical Characteristics of Patients by BMI Status.

	BMI<30 kg/m² (n=129)		BMI≥30 kg/m² (n=22)		p-value*
	Mean (Standard deviation)	Median (25th-75th percentile)	Mean (Standard deviation)	Median (25th-75th percentile)	
Age (years)	34.7(4.9)	35.0 (31.0-35.0)	33.9 (6.1)	34.0 (29.5-39.0)	0.521
Weight (kg)	62.5 (8.6)	61.0 (56.0-61.0)	91.9 (15.6)	94.5 (78.8-104.0)	<0.001
Height (m)	1.64 (0.1)	1.64 (1.60-1.64)	1.63 (0.1)	1.64 (1.60-1.69)	0.438
BMI (kg/m²)	23.1 (2.8)	22.9 (21.1-25.2)	34.3 (4.3)	32.5 (30.8-37.6)	<0.001
Infertility duration (years)	6.1 (4.2)	5.0 (3.0-7.0)	6.3 (4.9)	5.0 (3.8-7.3)	0.901
Number of previous trials	1.6 (0.8)	1.0 (1.0-2.0)	1.3 (0.9)	1.0 (1.0-1.0)	0.086
FSH (mIU/L)	7.7 (2.9)	7.2 (5.7-9.3)	6.3 (1.9)	7.0 (4.6-7.7)	0.032
LH (mIU/L)	5.5 (2.5)	4.9 (3.9-6.3)	4.7 (2.1)	4.4 (3.2-5.8)	0.148

*From t-test for normally distributed variables and Mann-Whitney test for non-normally distributed data (namely Infertility duration, Number of trials and LH levels).

Table 2. Descriptive Statistics of Ovulation Induction Factors of Patients by BMI Status.

	BMI<30 kg/m^2 (n=129)		BMI≥30 kg/m^2 (n=22)		p-value*
	Mean (Standard deviation)	Median (25th-75th percentile)	Mean (Standard deviation)	Median (25th-75th percentile)	
Number of days of stimulation	10.4 (1.4)	10.0 (10.0-11.0)	11.4 (1.2)	11.0 (10.0-13.0)	0.002
Total FSH dose (IU)	3410.9 (1239.0)	3375.0 (2400.0-4400.0)	4506.8 (1544.4)	4375.0 (3375.0-5850.0)	<0.001
E2 (day hCG)(pg/ml)	1961.6 (1192.0)	1666.0 (1109.3-2545.0)	1715.2 (1224.9)	1604.5 (876.3-2081.3)	0.274
No of follicles	7.6 (3.1)	7.0 (6.0-9.0)	7.0 (3.5)	7.0 (4.0-9.3)	0.457
No of oocytes	6.8 (3.0)	7.0 (5.0-8.0)	6.1 (3.3)	6.0 (4.0-9.0)	0.498
No of mature oocytes	4.4 (1.9)	4.0 (3.0-6.0)	4.5 (2.4)	4.0 (3.5-6.0)	0.692
Fertilization rate	0.67 (0.17)	0.67 (0.58-0.78)	0.69 (0.21)	0.67 (0.60-0.80)	0.598
Pregnancy (n (%))	23 (17.8)		9 (40.9)		0.023

*From t-test for normally distributed variables and Mann-Whitney test for non-normally distributed data and X2 test for pregnancy outcome.

Table 3. Genotype Distribution According to BMI Status.

Genotype Distribution	BMI<30 (N=129)	BMI≥30 (N=22)	Total (N=151)	p-value*
ESR1-PvuII				
TT	**32 (24.8%)**	**6 (27.3%)**	**38 (25.2%)**	**0.334**
TC	**72 (55.8%)**	**9 (40.9%)**	**81 (53.6%)**	
CC	**25 (19.4%)**	**7 (31.8%)**	**32 (21.2%)**	
ESR2-RsaI				
GG	**119 (92.2%)**	**22 (100%)**	**141 (93.4%)**	**0.401**
GA	**9 (7.0%)**	**0 (0%)**	**9 (6%)**	
AA	**1 (0.8%)**	**0 (0%)**	**1 (0.6%)**	
FSHR-Ser680Asn				
Ser/Ser	**28 (21.7%)**	**3 (13.6%)**	**31 (20.5%)**	**0.675**
Ser/Asn	**66 (51.2%)**	**12 (54.5%)**	**78 (51.7%)**	
Asn/Asn	**35 (27.1%)**	**7 (31.8%)**	**42 (27.8%)**	

* From t-test.

Finally, no differences were found concerning the gene polymorphisms distribution and the BMI status, when we compared the patients that were treated with the long and the short protocol.

DISCUSSION

This study is the first one to focus on the possible role of gene polymorphisms of hormonal receptors in obese non-PCOS women who enter an IVF/ICSI program. PCOS syndrome presents certain hormonal particularities, which affect the IVF and pregnancy outcome. In excluding PCOS patients, we aimed to observe the sole impact of obesity alone, without interfering factors such as high androgen levels associated in patients with PCOS, concerning all factors in ART. The polymorphisms studied were the PvuII polymorphism of ESR1 gene, RsaI polymorphism of ESR2 gene and Ser680Asn variation of FSH receptor, all linked with each other through various biochemical pathways. The FSH receptor activation activates the CYP19 aromatase gene *via* FSH hormone. The latter increases the oestradiol production, which in turn activates the ESR1 and ESR2 nuclear receptors [33, 34]. A recent study presented an indirect evidence of the involvement of the FSHR gene (or a locus close to it) in E2 production in a genome-wide linkage scan seeking quantitative trait loci involved in plasma levels of steroids [35]. On the other hand, the oestrogen levels influence FSH receptor

function *via* the adenylate cyclase system [36] and extend the action of FSH on granulosa cells by promoting their proliferation and increasing their expression of FSH receptors [37]. Excess adipose tissue, on the other hand, *via* leptin, increases peripheral aromatization of androgen to oestrogen and decreases the serum sex hormone-binding globulin (SHBG) levels enhancing the bioavailability of testosterone and oestradiol [38].

Our results suggest that the gene polymorphisms studied are not associated with increased BMI and that there is no over-presentation of a certain genotype in the obese non-PCOS population. The distribution of the genotypes of the three polymorphisms did not differ among obese and non-obese patients. The obese population studied is rather small, but comparable to the group of non-obese women. The retrospective character of the study did not permit the access to a larger number of obese patients. Nevertheless, the study supports that obesity does not appear to be an additional reason to perform a genetic control before IVF or ICSI, like poor response or failure in previous IVF/ICSI attempts do.

The other interesting finding of this study is the higher pregnancy rate observed in women with BMI≥30 kg/m² ($p=0.023$). This seems to be contradictory to other findings [14, 39] and reviews published [40] that showed decreased IVF pregnancy rates in obese women. One report, though, included live birth rates finding an increased percentage of early pregnancy loss in obese women [14] and the other two reports [39, 40] included obese patients with BMI > 25 or 27 kg/m². There may also exist differentiations among different nationalities or ethnic groups or populations with different dietary habits; the three reports refer to northern European populations. However, there are other reports that show that obese women have IVF pregnancy rates that are comparable with those of normal weight [10, 17, 41]. The pathophysiological mechanisms of the effect of increased BMI on IVF outcome is still not clear. One should also keep in mind the findings showing that uterine receptivity was unimpaired in women with increased BMI that entered anonymous oocyte donation, when hormonal support and embryo quality were standardized [42].

Women with BMI≥30 kg/m² needed an increased total FSH dose ($p<0.001$) and increased days of stimulation ($p=0.002$) in order to achieve a satisfactory oocyte number pick-up. The fact that the number of retrieved oocytes, as well as that of matured oocytes and the fertilization rate were comparable between the two groups is an indication that the increased FSH requirement is probably due to the excessive weight, and not a reduced threshold effect of exogenous FSH in obese women, as other investigators have underlined [43]. These findings agree with earlier observations showing that obese patients require a higher r-FSH dose than normal weight patients in order to achieve follicular maturation and that obesity does not affect negatively *in vitro* fertilization outcome [17].

An hypothesis explaining the higher pregnancy rate in the obese women group is the fact that this group also had lower basal FSH levels, compared with non-obese women, which may explain to an extend the better pregnancy outcome. The basal FSH levels are normal in both groups, but there is a significant difference between the groups, leading to the hypothesis that fluctuations of basal FSH levels may exercise a role in the IVF process. In previous studies in human oocytes and embryos, we showed that there are receptors of FSH as well as LH in the denuded oocytes and early-embryos developed from one-cell embryo to the blastocyst formation [44]. The expression of FSHR, regulated by FSH level, has a physiological role in follicular development. It seems that low basal FSH levels, up-regulating its receptors on the granulosa-oocyte complex, is positively associated with good quality retrieved oocytes. In our knowledge from literature, FSH level is a crucial biomarker for the outcome of pregnancy and concerning the quality of oocyte and embryo quality, especially in patients around 35 years old. In our study, from all biomarkers, as age and hormonal profile, the only marked difference was concerning the FSH level. AMH levels were not available for all patients, so it was not possible to use as a biomarker. Although new biomarkers are being used, FSH along with the age of the patient remains necessary biomarkers for the prediction of pregnancy and ovulation induction outcome [45].

Finally we tested the possibility that the difference in the number of subjects in the two groups may affect the difference in pregnancy rates, by applying a multiple logistic regression model, where the dependent variable in the model was pregnancy and the independent variables were: BMI, age, follicles, COC, Total FSH dose. The effect of obesity was positively and statistically significantly associated with gestation.

In conclusion, we did not observe differences in the genotype distribution of ESR1, ESR2 and FSHR gene polymorphisms studied when we compared the obese population with the non-obese population. Thus, obesity does not constitute an additional indication to perform a genetic analysis before entering an IVF/ICSI program. Nevertheless, there exist gene polymorphisms in obese women which seem to have a potential role as modulators of gonadal steroid function [46]. This is an indication that the research in that direction must continue in order to clarify the endocrinological mechanisms that associate obesity and reproduction and to be able to use them for the benefit of women trying to achieve a pregnancy.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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