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**ISSN No: 2319-5886**

**International Journal of Medical Research & Health Sciences, 2016, 5, 8:79-84**

# The frequency of genotypes for the SNP Ser/Ser in the studied population of Albanian women is higher in the Balkan region

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**ABSTRACT**

*In women undergoing natural cycles, just one oocyte is usually selected for ovulation, yet routine clinical techniques to support the development of multiple follicles using additional gonadotrophins result in numerous ovulations. Several parameters have been postulated as predictors of ovarian response (inhibin B, 17-β-estradiol and anti-Müllerian hormone). Nevertheless, the FSH level on the day 3 of menstrual cycle remains, the most widely used biomarker due to its low cost, although, the genetic background of individuals seems to determine the response of patients to rFSH stimulation better than the stimulation design. Consequently, the variants of FSHR were explored and they may be involved in the role of FSH receptor in mediated signal transduction and with ovarian response in infertile women submitted to ovarian stimulation. In this study we examined, for the first time, the prevalence of genotype variants Asn680Ser in population Albanian women from Kosovo Dukagjin region who took part in IVF / ICSI program. The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8%, respectively (Table 1). bE2 levels between the three genotype variants showed slight but statistically significant difference (p= 0.0308). No difference was also found between the genotype groups either in terms of AFC, amount of the FSH required for ovulation induction, stimulation length days, number of dominant follicles, oocyte retrieval number or endometrial thickness (Table 2). BMI was significantly higher in the Ser/Ser group as compared to those from the Asn/Ser or the Asn/Asn group (p= 0.0010) (Table 2). In the study population of Albanian women Dukagjin region of Kosovo had a higher incidence of Ser / SER genotype compared to Asn / Asn genotype. Our research results in the Albanian population differ from published data for other ethnic groups in the Balkans.*

**Key words**: FSHR, SNP, polymorphism, infertility, controlled ovarian stimulation(COS)

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## INTRODUCTION

The most important aspect of oocyte development is how it pertains to the generation of a healthy oocyte. The oocyte selected, ovulated and fertilized, is the cell that contributes all of the future embryo's organelles and cytoplasm while the sperm's contribution is purely genetic. Therefore, understanding how the ovulated egg is selected from all of the developing oocytes is of critical importance so that we can determine which to select for assisted reproductive technologies [ART]. In women undergoing natural cycles, just one oocyte is usually selected for ovulation, yet routine clinical techniques to support the development of multiple follicles using additional

gonadotrophins result in numerous ovulations. Furthermore, most of these eggs are capable of fertilization and normal embryonic development – therefore, the burning question is to define the difference between the one oocyte that is naturally selected for ovulation and those that we can support to ovulation [1]. Several parameters have been postulated as predictors of ovarian response. Since the ovarian function cannot be measured directly, the use of serum markers [inhibin B, 17-β-estradiol and anti-Müllerian hormone] and/or ultrasound variables [ovarian volume, measurement of antral follicles, ovarian stromal blood flow] have been proven to be useful, although limited [2]. Nevertheless, the FSH level on the day 3 of menstrual cycle remains, the most widely used biomarker due to its low cost, although, the genetic background of individuals seems to determine the response of patients to rFSH stimulation better than the stimulation design [3]. The response to FSH stimulation is determined by the number of follicles and their sizes, if there is a suspicion of OHSS, the dosage of estradiol is performed. The adequate response is determined by the presence of at least 4 follicles with at least 14 millimetres in size. Consequently, the variants of FSHR were explored and they may be involved in the role of FSH receptor in mediated signal transduction and with ovarian response in infertile women submitted to ovarian stimulation [4] was the first group that observed differences in Asn680Ser genotypes with dose of rFSH in COH. Other groups observed that the FSHR variants influence the basal FSH and estradiol levels with conflicting results [5], for this reason, the FSHR gene and others gene variations may play a role in modulating receptor sensitivity and intracellular second messenger cascades to exogenous hCG and other gonadotrophins [6], thus the FSHR SNPs may cause changes in the phosphorylation and glycosylation molecular mechanisms [7]. In vitro fertilization [IVF] is a complex multistep process that comprises collection of oocyte-containing follicles after controlled ovarian hyper stimulation [COH] with rFSH [recombinant Follicle-stimulation Hormone], oocyte fertilization, embryo development, embryo transfer to the uterus, and implantation. All these steps are critical for successful IVF. However, the initial critical step of this complex procedure is the COH, which aim is to safely obtain a high number of mature oocytes, as well as to allow the selection of the most viable embryo for transfer [8]. In this study we examined, for the first time, the prevalence of genotype variants Asn680Ser the population Albanians from Kosovo Dukagjin region who took part in IVF / ICSI program.

## MATERIALS AND METHODS

**Subjects.** The present study encompass 104 prospectively recruited female patients of Albanian ethnic population from Kosovo Dukagjin region enrolled in ICSI procedures at Polyclinic - IVF Centre, Peje, Republic of Kosovo in the period from January 2014 to February 2015. All patients were otherwise healthy women with functioning ovulation, and no history of endocrine diseases or known family history of inherited diseases. The reason of infertility in all cases has been identified as male factor. Informed consent was obtained from all the participants and the study was approved by the local Medical Ethics Rewiev Committee at Faculty of Medicine, University of Prishtina, Republic of Kosovo.

**Hormonal assays.** Serum levels (day 3 of the menstrual cycle) of FSH, LH, estradiol (E2), prolactin, progesterone, TSH, were measured by enzyme linked fluorescent assay (ELFA) using bioMerieux Mini Vidas Automated Immunoassay Analyzer (bioMérieux S.A. 69280 Marcy l'Etoile, France). The peak E2 levels were measured on the day of human choriogonadotropin (HCG) administration.

**Treatment.** In all cases, controlled ovarian stimulation was performed according to standard long protocol procedure as previously described. In general, transvaginal ultrasound (US) was performed to ascertain ovarian quiescence on the first 3 d of menses, and controlled ovarian stimulation was then initiated. Follicular development was monitored by transvaginal sonography, after 5 days of stimulation and then every other day. Exogenous FSH (Gonal F) is applied in a daily dosage of from three to six vials (225-450 IU), depending on the patient's previous or anticipated response, age, bFSH, bLH, antral follicle count (AFC) and body mass index. The gonadotropin-releasing hormone (GnRH) antagonist ganirelix 0.25 mg (Orgalutran, NV Organon, The Netherlands) was administered daily from the day the leading follicle reached a diameter of 14 mm to prevent premature luteinizing hormone rises and luteinization. Ovulation is triggered by the application of 10,000 IU of HCG (Pregnyl ®, NV Organon, The Netherlands), when at least two follicles scored 17-18mm in diameter and transvaginal ultrasound was performed to measure endometrial thickness. Oocytes retrieval was performed 34-36 hours after HCG injection by transvaginal ultrasound-guided follicle aspiration under mild sedation and analgesia. Luteal support is provided by progesterone (Utrogestan ®; Besins International, Montrouge, France), which was introduced after oocytes aspiration and extended to 8 weeks, or until the initiation of menses. Sperm and oocyte preparation, ICSI (intra cytoplasmic sperm injection) fertilization, embryo culture, and transfer were performed according to the conventional IVF laboratory guidelines. The clinical pregnancy was confirmed 1-2 weeks later by the transvaginal ultrasound detection of

gestational sac. Complete pregnancy follow-up of patients was performed by their private obstetricians in the majority of cases.

**DNA Isolation and genotyping.** Genomic DNA was extracted from 250 µL of whole blood using a PureLink™ Genomic DNA Mini Kit (Invitrogen, Life Technologies Corporation, Carlsbad, California, USA) according to the manufacturer's instructions. Analysis of the FSHR gene polymorphism at position 680 was carried out using predesigned TaqMan® SNP Genotyping Assay (rs6166; Life Technologies Corporation, Carlsbad, California, USA). Real-time PCR was performed using the TaqMan Universal master mix II and Applied Biosystems 7500 Real-Time PCR System (Life Technologies Corporation, Carlsbad, California, USA) in accordance with the manufacturer's instructions. The analysis was carried out in accordance with the instructions for the device used.

**Statistical analysis .** Statistical analysis, data were tested for normal distribution using D'Agostino-Pearson omnibus test. Variables are presented as the mean ± SD if distributed normally or as the median and range for nonparametrically distributed variables. Genotype and allele frequencies were analyzed by Chi-square test ($\chi 2$) test. Group comparisons were performed by the unpaired t test and one-way ANOVA (analysis of variance) or nonparametric Kruskal-Wallis and Mann-Whitney test for unpaired data. Correlation between two variables was ascertained by linear regression analysis and Pearson correlation coefficient calculation for parametrically distributed variables while Spearman's correlation test was used for nonparametrically distributed variables. The influence of two different variables on oocyte retrieval number was determined by two-way ANOVA analysis. Statistical analysis was performed by applying a commercially available software package (GraphPad Prism, GraphPad Software, San Diego, CA). All statistical tests were two-sided and a conventional value of $P < 0.05$ was used to represent statistical significance.

## RESULTS

A total of 104 patients underwent ICSI procedure and were genotyped in this study. The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8%, respectively (Table 1).

**Table 1. SNP Genotyping Asn680Ser FSH gene 104 patients in the study**

| SNP | Allele frequency% (n) | | Genotyps frequency % (n) | | Clinical pregnancy% (n) |
|---|---|---|---|---|---|
| Ser680Asn | A (N) | 45.67 (95) | AA (NN) | 22.12 (23) | 33.3(10) |
| | G (S) | 54.33 (113) | AG (NS) | 47.12 (49) | 43.3(13) |
| | | | GG (SS) | 30.77 (32) | 23.3(7) |

*number-n, Asparagine –A(N), Serin -G(S), Asparagin/Asparagin AA(NN), Asparagin/Serin-AG(NS), Serin/Serin-GG(SS).*

**Table 2. Clinical and endocrine characteristics of patients with respect to the SNP Asn680Ser FSHR gene**

| Clinical and endocrinologic parameters | NN (n=23) | NS (n=49) | SS (n=32) | p |
|---|---|---|---|---|
| Age (years) | 33.65 ± 6.278 | 34.06 ± 5.528 | 32.38 ±5.707 | 0.4308 |
| BMI (kg/m$^2$) | 22.00 (20.60-24.70)$^{*}$ | 22.30(20.20-25.10)$^{**}$ | 26.10 (22.88-27.70)$^{*,**}$ | **0.0010** |
| bFSH (mIU/ml) | 10.01 (7.60-12.16) | 10.20 (8.58-11.92) | 10.28 (8.32-12.18) | 0,8530 |
| b LH (mIU/ml) | 5.11 (4.56-5.82) | 5.56 (4.755-6.370) | 5.415 (4.553-5.878) | 0.3606 |
| bFSH/bLH | 1.976 (1.720-2.256) | 1.863 (1.666-2.181) | 1.961 (1.754-2.111) | 0.6532 |
| b Prolactin (ng/ml ) | 14.50 (7.20-20.00) | 17.40 (12.02-20.75) | 16.41 (9.50-20.58) | 0.2403 |
| b Estradiol (pmol/l) | 46.88 (36.75-55.09) | 42.90 (34.5-61.20) | 33.18 (26.25-45.53) | **0.0308** |
| b Progesteron (ng/ml ) | 0.740 (0.610-1.080) | 1.0 (0.780-1.205) | 0.840 (0.6755-1.035)) | 0.0855 |
| b TSH (uIU/ml) | 2.030 (1.14-2.67) | 1.76 (1.055-2.43) | 1.780 (1.183-2.313) | 0.7393 |
| AFC | 7.043 ± 1.965 | 7.122 ± 1.822 | 6.625 ± 2.379 | 0.5474 |
| FSH amount needed for ovulation induction (IU) | 2482 ± 467.8 | 2441 ± 532.7 | 2466 ± 481.5 | 0.9444 |
| Length of stimulation (days) | 12.57 ± 0.6624 | 12.51 ± 0.8447 | 12.66 ± 0.8654 | 0.7336 |
| Number of dominant follicles (d≥17mm) on the day of hCG administration | 4.565 ± 1.674 | 4.551 ± 1.542 | 3.813 ±1.693 | 0.1020 |
| Estradiol on hCG day of administration (pg / l) | 1746 ± 811.0 | 1782 ± 681.0 | 1467 ± 627.3 | 0.1234 |
| Endometrial thickness on hCG day of administration (mm) | 8.50 (8.00-8.80) | 8.40 (8.00-9.00) | 8.30 (7.725-8.875) | 0.6474 |
| Number of oocytes retrieved | 5.174 ±1.946 | 5.633 ± 1.845 | 4.906 ± 1.653 | 0.1997 |
| Number of transferred embryos | 2 (1-3) | 3 (2-3)$^{**}$ | 2 (1-2)$^{**}$ | **0.0101** |
| *Pregnancy rate* | *43.48 (10/23)* | *26.53 (13/49)* | *21.875 (7/32)* | 0.19398 |

*BMI Body mass index , bFSH= basale Follicle-stimulating hormone, bLH=,basaleLuteinizing hormone, AFC,=antral follicle count, bTSH=basale Thyroid-Stimulating Hormone, hCG=Human Chorionic Gonadotropin, bE2=basale Estradiol*

Values are presented as mean ± SD if the distributed parameter, or as median and range if they are distributed non-parametric. One-way ANOVA (Tukey multiple comparative test) was used to analyze the differences in terms of age, AFC, the amount of rFSH, length of stimulation, number of leading follicles (d≥14mm) on D-2nd, E2 to D-2nd, the number of oocytes retrieved and the number of transferred embryos. Chi-square analysis was used to analyze pregnancy. Kruskal-Wallis and Dunn's multiple comparative test was used to analyze non-parametric variables the difference. The value of $p<0.05$ was considered statistically significant. Values differ significantly between the groups (Dunn's multiple comparative test -see explanation in the text). NN - Asn / Asn, NS - Asn / Ser, SS - Ser / Ser; b stands for basal value; exogenous FSH (IU): amount required for appropriate hormonal stimulation of the ovaries; 2D-2: date for hCG to stimulate ovulation 36 hours before follicle aspiration. Clinical and endocrinologic parameters were analyzed based on the genotype variants (Table2), age, BMI, and oocyte retrieval number. The mean ages of the three genotype groups were similar and bFSH, bLH, bFSH/bLH, bE2, Prolactin, Progesteron, TSH. Although the overall analysis of bE2 levels between the three genotype variants showed slight but statistically significant difference ($p= 0.0308$). No difference was also found between the genotype groups either in terms of AFC, amount of the FSH required for ovulation induction, stimulation length days, number of dominant follicles, oocyte retrieval number or endometrial thickness (Table 2). BMI was significantly higher in the Ser/Ser group as compared to those from the Asn/Ser or the Asn/Asn group ($p= 0.0010$) (Table 2).

## DISCUSSION

Pharmacogenetic studies have revealed a series of genetic markers involved in COH response, markers of ovarian reserve and reproductive lifespan [9]. Among them, FSHR gene-associated SNPs, including the Asn680Ser missense variant, are the most promising genetic markers available to date. In the present study we investigated the association of Asn680Ser FSHR polymorphisms with the clinical and endocrinologic parameters of Albanian women from Kosovo Dukagjin region. Study revealed the dominant frequency distribution of the Asn/Ser genotype consistent with its highest rates found in other ethnic groups [3]. However, contrary to other ethnic population examined so far, the Ser/Ser variant in our population was represented in higher frequency compared to Asn/Asn homozygote. The frequencies of the Asn680Ser genotype variants were as follows: Asn/Asn 22.1%, Asn/Ser 47.1%, and Ser/Ser 30.8% [Table 1]. By all accounts, closure of the Albanian population, which is reflected low frequency of getting married with members of other ethnic groups, it could be reasonably determined by differences in the distribution of those SNP polymorphisms FSHR, compared to other ethnic groups. It seems that the social and cultural particularities preserve genetic parent "pool", which descended from the native indigenous population. Consistent with reported data present study also showed lower values for bFSH, and significantly higher AFC, peak E2 levels, and oocyte retrieval number in the youngest age group [10]. It has been reported that bFSH levels differ significantly among the Asn680Ser genotype variants with carriers of the Ser/Ser genotype having slightly higher bFSH levels and requiring a significantly higher gonadotropin dose to induce ovulation [3]. These studies suggest that the SS FSHR genotype variant is a factor of relative resistance to exogenous FSH stimulation. In agreement with previous reports in the present study the peak E2 levels, number of follicles, and the number of oocytes retrieved, as well as the clinical pregnancy rates were not significantly different among the genotype variants, suggesting that the treatment was equally successful, independent of the FSHR isoform [11]. However, the lowest values for AFC, the peak E2 levels, number of dominant follicle and oocyte retrieval number in our studied population were observed in Ser/Ser genotype group. Regarding the ovarian response, some studies report that Ser/Ser subjects in comparison to Asn/Asn or heterogeneous genotype variants have higher AFC, peak E2 levels and oocyte retrieval number and thus greater risk for OHSS [12]. Others report smaller oocyte retrieval number in Ser/Ser vs. Asn/Asn and/or Asn/Ser subjects thus suggesting quite contrary that the Ser/Ser genotype variant may be associated with a reduced ovarian response to COH [13]. In the present study Asn680Ser polymorphisms was significantly associated with bE2 levels in overall studied population, with the highest median level found among Asn/Asn and the lowest level in Ser/Ser group [Tabel 2]. These findings are in agreement with observations from previous studies that also indicate that elevated bE2 levels result in a greater number of oocytes and embryos for selection at the time of embryo transfer or cryopreservation without affecting pregnancy outcome [14]. BMI was also significantly associated with genotype variant subgroups in the present study with the Ser/Ser group showing the highest and Asn/Asn group the lowest BMI index. Therefore, to the best of our knowledge this is the first study showing the statistically significant association of BMI and Asn680Ser FSHR polymorphism[ Tabel 2]. It is already known that increased BMI may alter hormone metabolism and clearance in several complex ways [15]. Raised BMI was recently associated with adverse pregnancy outcome in women undergoing IVF/ICSI treatment. This effect was present in overweight [BMI ≥ 25] as well as obese women [16]. Except significantly higher prolactin levels in the overweight [BMI ≥ 25] group we did not find any statistically relevant difference between clinical and

endocrinologic parameters in the two BMI groups nor statistically significant correlation of BMI with other examined parameters in the overall population. The antral follicle count decreases over the years, is a predictor of the number of retrieved oocytes and can predict the likelihood of the IVF success [17]. Positive significant correlation between antral follicle count and number of retrieved oocytes and a negative correlation between antral follicle count and age was also observed in previous reports [18]. Although AFC values showed statistically significant correlation with oocyte retrieval number equally in overall population and all three genotype variant we found no statistically significant associations between the polymorphisms and this marker of ovarian reserve. No associations of FSHR genotypes with AFC were also described in previous study [19], [Table 2]. It should be noted that there is a great heterogeneity in the results that concern the efficacy of Asn680Ser FSHR genetic marker for the prediction of the ovarian response and pregnancy outcome. In the study population of Albanian women Dukagjin region of Kosovo had a higher incidence of Ser/Ser genotype compared to Asn/Asn genotype. Our research results in the Albanian population differ from published data for other ethnic groups in the Balkans.Therefore, these findings should be confirmed in larger studies, which will probably reveal more comprehensive results.

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