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Follicular fluid vascular endothelial growth factor is associated with type of infertility and interferon alpha correlates with endometrial thickness in natural cycle *in vitro* fertilization

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The aim of this study was to analyse the presence of vascular endothelial growth factor (VEGF) and interferon alpha (IFN-α) in the follicular fluid (FF) and their possible influence, as pro-angiogenic or anti-angiogenic factors, on *in vitro* fertilization outcome. The concentrations of VEGF and IFN-α were correlated with oocyte and embryo quality, concentrations of hormones in the serum, perifollicular blood flow and endometrial thickness. VEGF was detected in all FF samples (median 706.6 pg/ml, range 182.9-6638 pg/ml). IFN-α was detected in 60% of the samples (median 6.5 pg/ml, range 0-79.4 pg/ml), while in 40% of the samples its levels were below the test detection limit. VEGF and IFN-α concentrations did not correlate with the cause of infertility, concentrations of FSH, LH, E2 and prolactin, oocyte or embryo quality. Significantly higher concentrations of VEGF have been found in women with primary compared with secondary infertility ($p=0.011$, Mann Whitney test). The concentrations of VEGF and IFN-α did not correlate with the resistance index (RI) on days of hCG administration, follicular aspiration and embryo transfer. However, the concentrations of IFN-α correlated with endometrial thickness on the day of embryo transfer (Spearman correlation coefficient $\rho=0.4107$; $P<0.05$) but not on days of hCG administration and follicular aspiration. The mechanism of VEGF association with the previous ability of having a child needs to be clarified in future studies. The results of this study indicate a possible role of IFN-α in pathways of endometrial remodelling.

Keywords: follicular fluid, VEGF, IFN-α

1. Introduction

Human follicular fluid (FF) contains a variety of biological active products (hormones, growth factors, cytokines) known to affect follicular growth, oocyte viability and developmental potential and fertilization. Follicular fluid components are produced by both granulosa and theca cells, as well as resident and infiltrating leukocytes (1-3). Cytokines detected in the FF seem to derive from intrafollicular local production rather than from the peripheral blood entering the follicles through theca interna (4).

Follicular development as well as the formation and regression of the corpus luteum are accompanied with changes in the capillary network in the ovary. This process enables ovarian cells to obtain the oxygen, nutrients and precursors necessary to synthesize and release different hormones essential for maintenance of the ovarian function.

The VEGF, originally named vascular endothelial permeability factor, is a glycoprotein of molecular weight of approximately 45kDa (5). The VEGF family includes several members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PIGF), and two VEGF-like proteins.

The main function of VEGF is to regulate angiogenesis and vasculogenesis by stimulating endothelial cells proliferation, migration and survival. The role of VEGF in the ovary is to lead an angiogenic process which is critical for the follicular growth. However, Lam and Haines (6) have stated that VEGF plays more than a simple angiogenic role in the ovary as it is involved in a number of key events in the course of ovarian cycle including follicular growth, ovulation, corpus luteum development, and ovarian steroidogenesis. The authors have suggested that VEGF expression is under the regulation of gonadotropins or ovarian sex hormones. The main source of VEGF in the FF during the preovulatory stage are granulosa cells (4).

Type I interferons: IFN-α, IFN-β and IFN-ω, are most frequently found in humans. They have antiproliferative, apoptotic and anti-angiogenic effects and they modulate the immune response, specifically by activating dendritic cells, cytolytic T and NK cells. Preclinical data suggest that IFN-α anti-angiogenic activity may be associated with the regulation of endothelial cell motility (7) and survival (8), and with the inhibition of VEGF transcription (9).

The aim of this study was to investigate the role of VEGF and IFN-α in human FF as possible prognostic markers for assisted reproduction outcomes by correlating the concentrations of these biological response modifiers with hormones levels, oocyte/embryo quality, perifollicular blood flow, endometrial thickness or pregnancy.

2. Materials and methods

2.1. Patients' Characteristics

The study included 40 married couples who entered the natural cycle (NC) IVF/ET program at the Department of Obstetrics and Gynaecology, Sveti Duh Hospital, Zagreb, Croatia. The median age of female patients was 35 (range from 22 to 44).

The causes of infertility in the patients included tubal factor (n=14), male factor (n=12), unexplained infertility (n=2) and mixed cause of infertility (n=12).

The results of routine haematological, serological, microbiological and molecular tests (differential blood count, sedimentation, routine serological assays for blood donors, presence of anaerobic and aerobic bacteria, Human Papillomaviruses, *Chlamydia trachomatis* as well as *Ureaplasma and Mycoplasma* in cervical swabs) did not reveal acute or chronic infections in our patients. Endometriosis and pelvic inflammatory disease were excluded.

Written informed consent to use the FF samples was obtained during oocyte recovery by all patients. The Ethics Committee of the Hospital approved this study.

2.2. Blood collection and FSH, LH, E2, PRL analysis

Blood sample was collected on natural cycle Day 3 and centrifuged at 600x g for 10 min. Serum was stored at -20°C until quantification by electrochemiluminescence immunoassay (ECLIA) with the use of Elecsys 1010 (Roche Diagnostics, Minneapolis, MN, USA). The following kits were used: FSH reagent kit 1.775.863, CalSet FSH 1.775.880; LH reagent kit 1.732.234, CalSet LH 1.732.269; E2 reagent kit 1.776.002, 1.776.037 CalSet Estradiol; PRL reagent kit 1.775.952; 1.775.987 CalSet Prolactin. The reference values (median, range) for follicular phase are: FSH 6.9 IU/l (3.5 – 12.5); LH 5.9 IU/l, (2.4 – 12.6); E2 53.9 pg/ml, (24.5 – 195); PRL 218 mIU/l, (72 – 511).

2.3. Follicular fluid collection and cytokine assays

The study group included patients who underwent IVF procedure in the natural ovarian cycle and had one preovulatory follicle. After follicular aspiration and oocyte isolation, FF was taken and centrifuged at 600x g for 10 min, at room temperature. Supernatant was stored at -80°C. Quantification of VEGF and IFN-α in the FF was performed at the Department of Immunological and Molecular Diagnostics, University Hospital for Infectious Diseases "Dr. Fran Mihaljević", Zagreb, Croatia, according to the manufacturer's instructions. The concentrations of VEGF in FF were determined by the commercial Human VEGF Immunoassay kit (Bio Source International Inc., Camarillo, CA, USA). The minimal and maximal detectable amount for VEGF was <0.5 pg/ml and 1500 pg/ml, respectively. The concentrations of IFN-α in FF were determined by the commercial enzyme immunoassay Quantikine Human IFN-α Immunoassay (R&D Systems, Minneapolis, MN, USA). The minimal and maximal detectable amount for IFN-α was <10 pg/ml and 500 pg/ml, respectively.

2.4. Ovarian blood flow measurement and ultrasound assessment of the endometrium

Forty patients in the natural cycle underwent transvaginal ultrasound follow up of follicular development from natural cycle Day 8. Ultrasound device (Aloka, Tokyo, Japan) was equipped with a 5-MHZ frequency transvaginal transducer that was used for follicle measurement and blood flow assessment by color and pulsed wave Doppler. When the follicle reached 18 mm in diameter, ovulation was triggered by the administration of 5000 IU human chorionic gonadotropin - hCG (Choragon, Ferring Pharmaceuticals Ltd, GmbH, Kiel, Germany).

Blood flow of the dominant follicle, the same one from which the FF was obtained, was examined for the presence of color signals indicating vascularization, while the blood

flow assessment was expressed as resistance index (RI). RI was defined as (PSV-EDV)/PSV (peak systolic velocity, PSV; end-diastolic velocity, EDV) and higher RI value indicated lower blood flow. The spatial peak temporal average intensity for B-mode and Doppler imaging was below 95 mW/cm^2. Data of RI were recorded on the day of administration hCG, on the day of follicular aspiration and on the day of embryo transfer.

The B-mode ultrasound was used to evaluate thickness and echogenicity of the endometrium on the day of hCG administration, on the day of follicle aspiration and on the day of embryo transfer. The endometrium was depicted in the sagittal plane and measured as a bilayer thickness from the proximal myometrial-endometrial junction to the distal myometrial-endometrial junction.

2.5. In vitro fertilization - embryo transfer procedure

After recovery, the oocyte was washed free from the FF. Oocyte maturity (i.e. quality) was assessed after mechanical dissection of cumulus oophorus till corona radiata. The oocyte was pre-incubated for 4 hours in the Quinn's Advantage Fertilization medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization, Inc., Trumbull, CT, USA) at 37°C in 5.8% CO_2 and humidified air.

Semen samples were collected on the day of follicle aspiration. The semen samples were centrifuged at 300x g in the Quinn's Sperm Washing Medium (SAGE In Vitro Fertilization) and subsequently processed by the swim-up method in the Quinn's Advantage Fertilization medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization). Each oocyte was inseminated with $40x10^3$ to $80x10^3$ of motile sperm. Fertilization, as the presence of pronuclei, was checked about 20 hours after insemination and zygotes were placed into the Quinn's Advantage Cleavage medium with 5.0 mg/ml HSA (SAGE In Vitro Fertilization). If

oocytes did not contain pronuclei, the fertilization was checked once more after another 24 hours.

Embryos were graded according to the Istambul consensus (10). On culture Day 3, after ultrasound evaluation of the uterus and ovaries, only a single embryo has been transferred. Luteal phase support has been accomplished with the micronized progesterone (Utrogestan; Laboratories Piette International S.A., Brussels, Belgium) 600 mg/day starting from the day after oocyte retrieval.

Biochemical pregnancy test was performed two weeks after follicular aspiration, and clinical confirmation of pregnancy was performed by sonography after 6 weeks of gestation. Clinical pregnancy with foetal cardiac activity was described as a viable intrauterine pregnancy. Clinical pregnancy rate was expressed as the number of clinical pregnancy per embryo transfer. The term IVF outcome referred to the live birth rate per embryo transfer.

2.6. Statistical analysis

Statistical analysis was performed using SAS (version 6.12, SAS Institute, Cary, North Carolina, USA). D'Agostino-Pearson normality test was used to assess the normality of distribution. Normally distributed variables were described by mean ± standard deviation, while variables that were not normally distributed were shown as median and interquartile range. For parameters without normal distribution, test of significance between groups was performed using the Mann Whitney test or Kruskal-Wallis test. Friedman ANOVA was used for testing the difference among repeated measurement. Pearson's correlation coefficient was applied to measure the degree of association between the parametric variables, and Spearman's ρ for nonparametric variables. A P-value of <0.05 was considered as statistically significant.

3. Results

3.1. IVF results

Out of 40 follicular aspirations in natural cycles we detected and isolated oocytes from the FF in 33 cases. The general observations of oocytes and embryos were as follows: out of 33 oocytes, 28 have reached meiotic maturity (metaphase II), 2 oocytes were immature (in the germinal vesicle stage), and 3 oocytes were atretic. *In vitro* fertilization of 28 oocytes resulted in the development of 25 two-pronucleated zygotes, while in two cases zygotes were three-pronucleated. The fertilization rate was 96.4%. As to embryo morphology, 10 cleaving embryos were grade I, 8 embryos were grade II, 5 embryos were grade III and 2 were grade IV at the day of embryo transfer. Biochemical pregnancy test was positive in 8 patients. Clinical pregnancy was detected in 7 patients and clinical pregnancy rate per embryo transfer was 21.1%. All clinical pregnancies resulted in live births.

3.2. Serum concentrations of hormones

Mean basal serum level of FSH was 5.7 IU/L (standard deviation SD±1.53). Median concentration of LH was 3.6 IU/L (range 1.6-14.3); E2 was 148 pg/mL (range 81-659); and prolactin was 311 mU/L (range 121-1535).

Regarding the presence of oocyte in FF and IVF outcome, serum concentrations of analysed hormones did not show statistically significant differences (Mann Whitney test).

3.3. Ovarian blood flow

Peri-follicular blood flow on the day of hCG administration, expressed as a RI mean value, was 0.49 (SD±0.06), while on the day of follicular aspiration the median RI value was 0.44 (range 0.35-0.74). Mean RI of peri-luteal blood flow on the day of embryo transfer (n=25) was 0.42 (SD±0.04).

There was significant (Friedman ANOVA P=0.003) decrement in RI values between the day of hCG administration and the day of follicular aspiration. There was no significant difference between RI values among patients regarding the presence of oocyte in FF and IVF outcome (Mann Whitney test).

3.4. VEGF

VEGF was detected and measured in all FF samples (n=40), concentrations were between 182.9 pg/ml and 6638 pg/ml (median 706.6 pg/ml).

The concentrations of VEGF did not significantly correlate with the serum hormone levels, RI of peri-follicular or peri-luteal blood flow as well as with endometrial thickness (Spearman correlation).

Significantly higher concentrations of VEGF have been found in women with primary compared with secondary infertility (p=0.011, Mann Whitney test) (Figure 1.). No significant differences in VEGF concentrations were found in patients analysed according to the oocyte presence in the FF, oocyte quality, fertilization, embryo quality or IVF outcome (Mann Whitney test) or cause of infertility (Kruskal Wallis test).

3.5. IFN-α

IFN-α was detected in 24 FF samples and measured in range from 0 pg/ml to 79.4 pg/ml (median 6.53 pg/ml), while in 16 samples its concentration was below the test detection limit.

The concentrations of IFN-α did not significantly correlate with the serum hormone levels, RI of peri-follicular or peri-luteal blood flow (Spearman correlation).

Regarding endometrial thickness, there was no correlation between IFN-α and endometrial thickness on the day of hCG administration as well as on the day of follicular aspiration (Spearman correlation). However, there was a statistically significant correlation

between IFN-α concentration and endometrial thickness on the day of embryo transfer (Spearman correlation coefficient $\rho=0.4107$; $P<0.05$) (Figure 2).

No significant differences in the FF IFN-α concentrations were found in patients analysed according to the oocyte presence in the FF, oocyte quality, fertilization, embryo quality or IVF outcome (Mann Whitney test) or the cause of infertility (Kruskal Wallis test).

3. Discussion

In this study we used the model of natural IVF cycle. We have followed up a single follicle and evaluated the concentration of FF VEGF (proangiogen) and IFN-α (antiangiogen) with regard to the cause of infertility, basal serum hormone levels, peri-follicular and peri-luteal blood flow, oocyte presence and quality, fertilization, embryo quality, endometrial thickness and IVF outcome.

Cunha-Filho et al (11) have found that intrafollicular VEGF concentrations were between 1020 and 1560 pg/ml in patients included in the NC IVF procedure. Contrary to these findings, the levels of VEGF in our study were below 1000 pg/ml in the majority of patients. The difference in the results of the two studies could be, at least in part, explained by the use of enzyme immunoassays from different manufacturers.

Kollmann et al (12) have analysed serum and FF cytokine concentration of patients included in stimulated (c-IVF) and NC IVF procedure. Using multiplex analysis system for cytokine/growth factor quantification in the FF (c-IVF=31; NC IVF=15) and serum (c-IVF=21; NC IVF=12), the authors showed that ovarian stimulation influenced the cytokine concentration in the FF and serum. Concentrations of cytokines and growth factors (including VEGF) were higher in the FF compared to the serum. In the natural cycles, the difference between cytokine concentration in the FF and serum was more pronounced compared to the stimulated cycles. The authors suggested that exogenous gonadotrophins probably induce the intrafollicular release of cytokines and chemokines, which leads to an increase of endothelial permeability.

Monteleone et al (13) have shown a direct positive correlation between FF VEGF concentration and peri-follicular vascularity grading on the day of follicular aspiration in hormonal stimulated IVF normoresponder patients. Since VEGF is secreted from granulosa and theca cells, it is likely that higher VEGF levels reflect a greater ability of the follicle to

create its own vascular network and therefore guarantee a better follicular microenvironment for the developing oocyte. However, our study, conducted on patients included in the NC IVF, failed to demonstrate a correlation between the FF concentration of VEGF and peri-follicular blood flow, but it demonstrated a statistically significant increase in the peri-follicular blood flow between the day of hCG administration and the day of follicular aspiration. Our findings suggest that perifollicular blood flow has a tendency to improve follicular supply from the follicular to the periovulation phase of cycle.

Kim et al (14) have shown that with the follicle size increasing, the follicular blood flow and FF VEGF concentration increase as well. However, only follicular blood flow was positively associated with pregnancy outcome. These results suggest that follicular blood flow might be an effective prognostic marker of IVF pregnancy outcome.

It was shown that oocytes obtained from follicles with higher grade of vascularization showed a higher rate of fertilization, better quality embryos and higher pregnancy rates (13). It is likely that a higher grade of perifollicular vascularity determines better cytoplasmic biochemical conditions that are not evaluated with routine parameters of oocyte quality (15).

Lee et al (16) reported that serum VEGF concentration at 11-14 days after ET showed a positive correlation with follicular VEGF concentrations and were significantly higher in pregnant women compared with non-pregnant women. Ntala et al (17) reported that VEGFR2 differed significantly between two groups (pregnant and non- pregnant patients), being higher in the pregnancy group. Contrary to these findings, our study failed to show a correlation between the concentration of VEGF in the FF and IVF outcome. Our findings are concordant with the findings from Benifla et al (18) who suggested that concentrations of VEGF in the FF could not predict assisted reproductive outcomes, especially among patients younger than 40 years. Van Blerkom et al (15) reported that FF VEGF could be an indicator for a healthy

follicle but that it cannot be used as a clinical prognostic marker for pregnancy outcome in assisted reproduction.

Interestingly, our results have shown that FF VEGF concentrations were significantly higher in patients with primary sterility in comparison with secondary sterility patients. Asimakopoulos at al, (19) Ocal et al (20) and Friedman et al (21) showed an association between elevated FF VEGF levels and diminished pregnancy potential. The authors suggested that a relative follicular hypoxia could be the reason behind the increased FF VEGF concentrations and poor oocyte competence. The main function of VEGF is to control the new blood vessels formation and the protection of endothelial and granulosa cells (22). The mechanism which is responsible for VEGF synthesis has not been completely elucidated. However, literature data suggest that hypoxia and hypoglycemia are the main stimulae of VEGF synthesis (23, 24). Besides hypoxia, other factors may influence the FF VEGF concentration as well (25). For example, the role of bone morphogenetic proteins (BMP) in ovarian physiology (particularly BMP-7) has been recently investigated as well (26).

The sources of FF VEGF are granulosa cells and FF macrophages (27). Based on our results, there was no correlation among FF VEGF, serum gonadotropins levels and sex hormones levels in patients included in the NC IVF procedure. *In vitro* models have shown that VEGF induced proliferation of granulosa cells and increased proliferative response of these cells to FSH, but VEGF alone or in combination with FSH had no effect on the expression of the steroidogenic enzyme (28).

The influence of proangiogenic factors is counterbalanced by a number of inhibitory agents. The net result of these opposing factors on the vascular endothelial cell determines the outcome of angiogenetic homeostasis. IFN-α is a naturally occurring protein that has the ability to decrease the transcription of VEGF gene expression through an Sp1 and/or Sp3-dependent inhibition of VEGF promoter activity *in vitro* (29). *In vivo* studies demonstrated

that the antitumor effect of recombinant IFN-ß may be mediated, at least in part, via angiogenesis inhibition rather than antiproliferative activity (30) and that IFN-α and retinoic acid have remarkable synergistic antiangiogenic effects (31,32).

Sarapik et al (33) analysed pro-inflammatory cytokines and chemokines using flow cytometry in individual FF of 153 c-IVF patients. Similarly to our results, IFN-α ranged between zero and 161.9 pg/ml. The authors have found that the healthy women (male factor infertility) had higher levels of FF IFN-α and follicular diameter was positively associated with IFN-α concentration. By using mRNA analysis, they have shown the presence of IFN-α in the mural and cumulus granulosa cells. The authors have suggested that this finding supports positive role of IFN-α in reproduction.

Baskid et al (34) studied FF and plasma cytokine profiles in 10 patients included in the NC IVF procedure by multiplex immunoassay. Despite the limited number of patients, their results confirm the presence of IFN-α in the FF of patients included in the NC IVF.

IFN-α induces the transcription of hypoxia-inducible factor-1a (HIF-1a) to inhibit the proliferation of human endothelial cells (35). According to our study, intrafollicular level of IFN-α was not associated with the perifollicular blood flow in the periovulatory period.

Our results have shown a positive correlation between IFN-α concentration and endometrial thickness. The results suggest that IFN-α has a role in pathways related to endometrial remodelling. There are conflicting data whether IFN-α affects the quantity of estrogen receptor with both increase and decrease in the receptor expression (36-39). Studies in patients with sequential treatment of IFN and tamoxifen showed an increase in hormone receptors and P24 protein (estrogen-regulated protein) (40). Panchanathan et al (41) have shown that activation of IFN-α signalling in mouse immune cells up-regulates the expression of estrogen receptor-alpha and E2. These findings support the hypothesis that female sex hormone estrogen and increased levels of IFN-α act via mutually positive feedback loop *in*

vivo. Estrogen is a biological parameter which enhances the endometrial growth. The association between IFN-α and E2 *in vivo* possibly also contribute to the sex biased autoimmune diseases, such as systemic lupus erythematosus, that predominantly affect women of childbearing age. Possible IFN-α enhancement of endometrial quality needs future investigation.

Our findings have indicated that peri-follicular blood flow has a tendency to improve follicular supply from the follicular to the periovulation phase of the cycle, but without correlation with FF IFN-α and VEGF. An association between FF VEGF concentration and previous ability of having a child as well as the possible role of IFN-α in pathways of endometrial remodelling need to be clarified in future studies.

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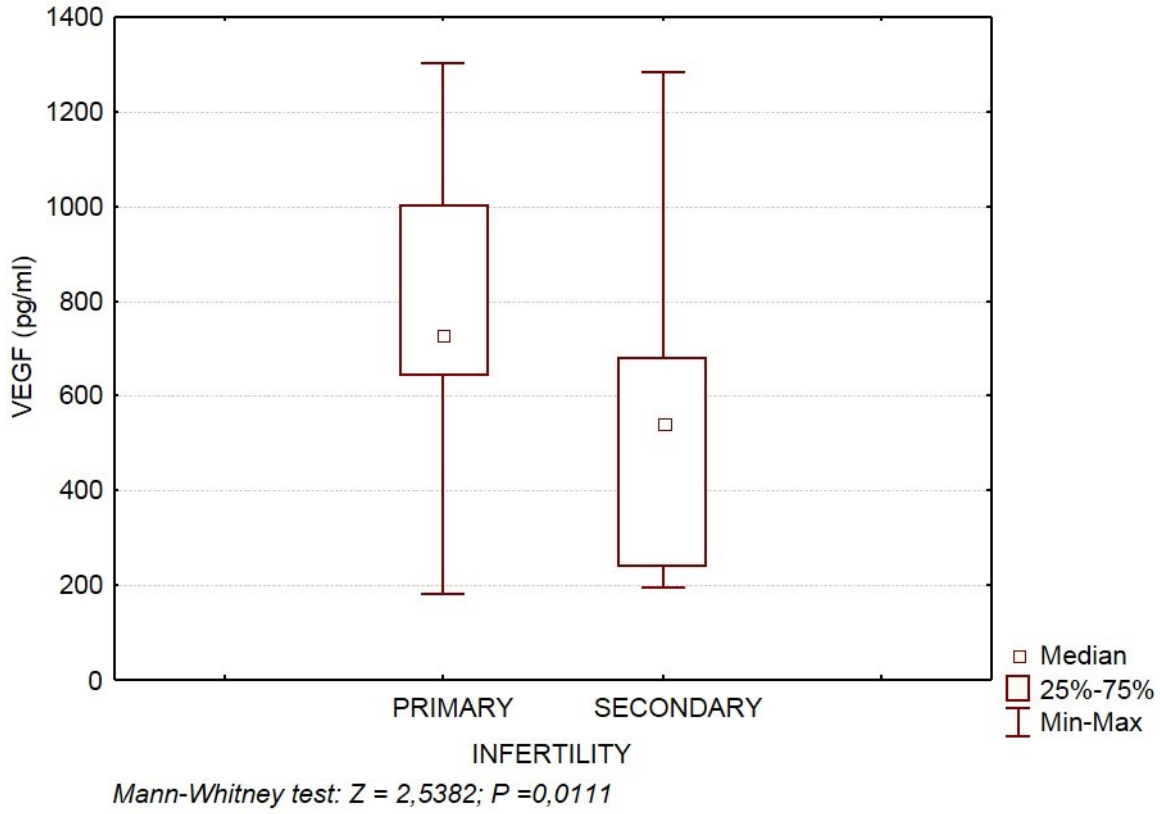
Figure 1. Association between follicular fluid VEGR and type of infertility



Mann-Whitney test: Z = 2,5382; P =0,0111

Figure 2. Correlation between follicular fluid IFN-α and endometrial thickness on the day of embryo transfer