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HIGH LEVELS OF SOLUBLE VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 1/SFLT1 AND LOW LEVELS OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN FOLLICULAR FLUID ON THE DAY OF OOCYTE RETRIEVAL CORRELATE WITH OVARIAN HYPERSTIMULATION SYNDROME REGARDLESS OF THE STIMULATION PROTOCOL

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The aim of the study was to assess the predictive value of vascular endothelial growth factor (VEGF), its soluble receptor - sVEGF-R1/sFlt1 and endocrine gland-derived vascular endothelial growth factor (EG-VEGF) concentrations in serum and follicular fluid (FF) for ovarian hyperstimulation syndrome (OHSS) in women undergoing controlled ovarian hyperstimulation (COH) protocols. Patients have been divided into 3 groups: control group on natural cycle, patients stimulated with GnRH agonist and patients stimulated with GnRH antagonist. The FF and serum concentrations of VEGF, EG-VEGF, sVEGF R1 and the expression of VEGF and EG-VEGF mRNA in GC in small and large follicles collected from patients were investigated. When we compared all patients in a trial, OHSS occurrence was correlated with higher level of sVEGF R1 and a lower level of VEGF in a follicular fluid from large follicles in a day of oocyte retrieval. The VEGF/sVEGF-R1 ratio for patients in COH groups, above which the risk of developing OHSS is very low (OR 0.1 (95% CI 0.01 – 0.29, P = 0.0006) was found to be 0.281 pg/ml, with AUC – 0.738, P = 0.042, (95% CI 0.656 – 0.82). High levels of sVEGF-R1 and low level of VEGF in FF on the day of oocyte retrieval correlate with OHSS regardless of the stimulation protocol.

Key words: *vascular endothelial growth factor, endocrine gland-derived vascular endothelial growth factor, soluble receptor, ovarian hyperstimulation syndrome, controlled ovarian hyperstimulation*

INTRODUCTION

Ovarian hyperstimulation syndrome (OHSS) is potentially life-threatening condition. In its severe form it is rare, as it occurs in 1 to 2% *in vitro* fertilization (IVF) cycles, however in mild form it might occurs in 40% of cycles (1, 2). This condition is associated with increased vascular permeability, which leads to transition of the intravascular fluid into the third space, which results in hemoconcentration and the risk of thromboembolism. Around 30 factors are involved in the regulation of cellular permeability (3). The vascular dysfunction caused by vasoactive substances released by the ovary appears important in OHSS (4), with VEGF being the most significant (5-9). VEGF exerts its role by binding to specific receptors situated in endothelial cells called VEGF-R1 (Flt-1) and VEGF-R2 (KDR) (10). It has been shown that a soluble variant of VEGFR-1 (sVEGF-R1/sFlt-1) is generated by differential splicing of the *flt-1* mRNA. Soluble VEGF-R1 is a naturally produced decoy receptor capable of binding and sequestering VEGF and is able to reduce the level of free, active VEGF (11). The soluble receptor is mainly complexed with ligands. Only 5 – 10% remains detectable as free, uncomplexed receptor protein (12).

Endocrine gland-derived vascular endothelial growth factor (EG-VEGF) has similar biologic functions as vascular endothelial growth factor (VEGF), however it does not show any structural homology to the VEGF family (13). It induces tissue specific proliferation, survival, migration and fenestration in capillary endothelial cells and its expression is mainly restricted to steroidogenic glands (ovary, testis, adrenal gland and placenta) (14, 15). Until today no soluble receptor for EG-VEGF has been identified, and sVEGF-R1 and R2 do not interfere with this protein. EG-VEGF acts through its two G-protein-coupled receptors PRK1 and PRK2 (prokineticin receptor 1 and 2). Angiogenic effect of EG-VEGF appeared to be restricted to endothelial cells derived from endocrine tissues (13). The cellular localization and temporal and spatial expressions of EG-VEGF in ovarian tissue are complementarily correlated to VEGF (16, 17), suggesting that EG-VEGF and VEGF participate synergistically in angiogenesis (17-19). Both proteins act additively or in a cooperative response on adrenal cortex derived capillary endothelial cells (ACE) in inducing fenestration, and no effect was observed in the absence of VEGF or EG-VEGF.

In order to find the predictive factors, if any, for OHSS, FF and serum levels of VEGF, EG-VEGF, sVEGF-R1 were investigated in this study. FF and serum levels of VEGF, EG-VEGF and sVEGF-R1 were measured in patients under both COH protocols and compared with patients during IVF procedure without COH (natural cycle) and between both COH groups. We also compared levels of VEGF, EG-VEGF and sVEGF-R1 with OHSS occurrence to identify correlations between them and the incidence of OHSS. The expression of VEGF and EG-VEGF mRNA in granulosa cells (GC) was also measured.

MATERIALS AND METHODS

Patients

The study was approved by the Bioethical Committee of Central Clinical Hospital of Interior in Warsaw (Poland) (67/2011) and informed consent for the collection and storage of serum, FF and GC, which would not affect the IVF/ICSI procedure, was obtained from all patients in this study.

This observational retrospective case control study was conducted in 'Novum', Fertility Clinic (Warsaw, Poland), where 148 infertile women were treated with IVF/ICSI (intracytoplasmic sperm injection). Patients were enrolled in the study from January 2012 to February 2015. The study was approved by the Bioethical Committee of Central Clinical Hospital of Interior in Warsaw (Poland) (67/2011) and informed consent for the collection and storage of serum, FF and GC, which would not affect the IVF/ICSI procedure, was obtained from all patients in this study.

Patients were divided into three groups. The control group consisted of women prepared to undergo IVF procedure with no stimulating medicament. Two treatment groups were patients in GnRH agonist protocol or GnRH antagonist protocol. The patient's characteristics are presented in *Table 1*.

Ovarian stimulation and triggering of final oocyte maturation

In a natural cycle group, after at least one dominant follicle reached the diameter of 17 mm hCG (Pregnyl; MSD, Oss, the Netherlands) was administered.

In treatment groups a flexible GnRH antagonist protocol or a long GnRH agonist protocol was used for controlled ovarian stimulation, as previously described (20). Shortly, in GnRH agonist long protocol, after pituitary suppression with triptorelin (Gonapeptyl®, Ferring GmbH, West Drayton, UK or Diphereline SR®, Ipsen Pharma, Boulogne Billancourt, France), which started in the midluteal phase of preceding cycle, ovarian hyperstimulation was performed by administration of both menotropin (Menopur®, Ferring GmbH, West Drayton, UK) and rFSH (Gonal-F; Merck Serono, Geneva, Switzerland). The starting doses of menotropin ranged from 75 to 150 IU/day and for rFSH it was between 37.5 IU and 225 IU/day. The GnRH antagonist (short protocol) multiple-dose protocol involved the administration of 0.25 mg GnRH antagonist cetrorelix acetate (Cetrotide®, MerckKGaA, Darmstadt, Germany) daily when the dominant follicle was about 12 – 13 mm in diameter until hCG administration. Follicle size was monitored serially by transvaginal ultrasound (GE Voluson, 6, 5 MHz transvaginal probe). The gonadotropin dose was adjusted according to the patient's response, measured by sequential transvaginal ultrasonography and the serum E2 levels. The starting doses of menotropin (Menopur®, Ferring GmbH, West Drayton, UK) ranged from 75 to 150 IU/day and for rFSH (Gonal-F; Merck Serono, Geneva, Switzerland) it was between 37.5 IU and 225 IU/day. HCG (Pregnyl®; MSD, Oss, the Netherlands) was administered in doses between 5000 IU at the presence of at

Table 1. Patient's characteristics.

	Control (n=48)	**GnRH agonist** (n=49)	**GnRH antagonist** (n=51)	**P-value control vs. GnRH agonist**	**P-value control vs. GnRH antagonist**	**P-value GnRH agonist vs. GnRH antagonist**
Age (years ± S.D.)	37.4±5.11	32.8±3.33	34.4±3.81	0.00001*	0.006*	0.04*
Years of infertility (years ± S.D.)	4 ± 1.82	3.4±1.21	3.9±1.62	0.43	0.54	0.99
Nulligravida (n/%)	20/41.7	38/77.6	25/49	0.0003*	0.46	0.03*
Nulliparae (n/%)	28/58.3	11/22.4	26/51	0.0003*	0.46	0.03*
Multigravidae (n/%)	13/27.1	5/10.2	7/13.7	0.03*	0.07	0.59
First IVF (n/%)	10/20.8	39/79.6	23/45.1	P<0.001*	0.08*	0.0003*
Second IVF (n/%)	11/22.9	9/18.4	13/25.5	0.62	0.73	0.39
Third or more IVF (n/%)	27/56.3	1/2	15/29.4	P<0.001*	0.007*	0.0001*
Previous OHSS (n/%)	2/4.2	0/0	6/11.8	P<0.001*	0.051	P<0.001*
FSH (mIU/ml ± S.D.)	9.31±4.39	6.36±1.7	7.08±2.92	P<0.001*	0.005*	0.38
AMH (ng/ml ± S.D.)	0.67±1.22	2.57±2.12	2.17±3.18	0.71	0.24	0.3
Estradiol at the day of hCG administration (pmol/l ± S.D.)	923.45±441.25	5770.39±2710.17	5402.51±2688.85	P<0.001*	P<0.001*	0.55
Progesteron at the day of hCG administration (ng/ml ± S.D.)	1.43±1.28	8.95±5.95	7.18±5.13	P<0.001*	P<0.001*	0.07
BMI (kg/m^2 ± S.D.)	22.19±2.85	22.66±3.03	22.58±3.5	0.99	0.47	0.97
Number of follicles at retrieval ± S.D.	2.33±1.47	16.2±8.84	12.82±6.60	P<0.001*	P<0.001*	0.86
Small follicles at retrieval ± S.D.	1.85±1.04	5.6±3.99	7.1±5.4	0.00004*	0.00001*	0.24
Large follicles at retrieval ± S.D.	1.57±1.04	7.9±5.11	6.17±3.53	P<0.001*	P<0.001*	0.06

*- statistical significance

least three follicle of ≥ 17 mm in diameter along with the increase in serum E2 concentration. All patients were triggered with hCG.

Oocyte retrieval and in vitro fertilization procedures

Transvaginal ultrasound-guided oocyte retrieval was performed 36 hours after hCG administration. On a day of oocyte retrieval blood sample of 15 ml was collected from a patient, and serum after preparation was stored at –80°C for further analysis.

The FF was aspirated separately from each follicle during oocyte retrieval. Any follicle aspirates that were not clear or contaminated with blood were discarded. Follicles were divided into two groups depending on the size measured in ultrasonography on a day of oocyte retrieval. Small follicles were smaller or equal than 17 mm and large follicles were bigger than 17 mm but smaller than 25 mm in diameter. The upper diameter of the follicle for oocyte collection along with follicular fluid was 25 mm according to IVF procedures, as bigger follicles mostly do not contain any oocyte or a poor quality oocyte. The follicular fluid from the follicles larger than 25 mm was aspirated in order to prevent ovarian cyst formation following ovarian stimulation and was not pooled with other FF from the same patient, FF and GC from follicles from a single patient were pooled. FF was immediately centrifuged without any medium for 2 min at 2000 g and the supernatants were stored. After oocyte retrieval GC was isolated and stored at –80°C for further analysis.

Intracytoplasmic sperm injection was performed in all cases. Embryo transfer was performed 3 – 5 days after oocyte aspiration.

Severity of OHSS was classified according to Golan classification (21). Women were monitored for development of OHSS by measuring haematocrit and white blood cells (WBC) on the day of oocyte retrieval and on day 7 POR (post oocyte retrieval), as well as by ultrasound assessment of ovarian size and ascetic fluid on day 7 POR.

Laboratory assays

The concentration of VEGF, EG-VEGF and sVEGF-R1 in serum and FF was determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (MaxDiscovery, Bioo Scientific, USA for VEGF, Nordic BioSite, Sweden for EG-VEGF and Quantikine, R&D System, Minneapolis, USA for sVEGF-R1). The samples were prepared and tested in duplicate according to the manufacturers' instructions. The concentrations were expressed as pg/ml. The assay sensitivity was 5 pg/ml. The inter- and intra-assay coefficients of variation were < 7 and < 4.5%, respectively.

The total RNA was isolated from GC using Total RNA Mini Kit (A&A Biotechnology, Poland) according to manufacturer's instruction. Isolated RNA was reversed to cDNA using VerteKIT (Novazym, Poznan, Poland) with oligo$(dT)_{15}$ primers according to manufacturer's instruction. The concentration of cDNA was analyzed by NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc. Waltham MA, USA). Real-time PCR was performed using a LightCycler 1.5 Instrument and LightCycler®FastStart DNA Master SYBR Green I detection kit (Roche Applied Science, Basel, Switzerland) as per the manufacturer's protocol. The final 20 µl real-time PCR reaction included 500 ng RT product, 1 µl of each primer, 0.8 µl $MgCl_2$ and 2 µl of LightCycler®FastStart DNA Master SYBR Green I. To reach a total volume of 20 µl per capillary, sterile water was added.

The thermal cycling conditions were 95°C for 10 min, followed by 45 cycles at 95°C for 15 s for denaturation, 60°C for 60 s for annealing and 72°C for 15 s for extension. After 45 cycles, a melting curve was generated by slowly increasing (0.2°C/s) the temperature from 70°C to 99°C, while the fluorescence was measured. As a reference, the expression of *β-actin* gene was quantified for each sample. The reactions for *VEGF*, *EG-VEGF* and β-actin were carried out in separate tubes. The results were analyzed by calculation formula: folds=$2^{-\Delta\Delta Ct}$, for *VEGF*: ΔΔCt=[Ct *VEGF* – Ct *β-actin*]$_{\text{experiment sample}}$ – [Ct *VEGF* – Ct *β-actin*]$_{\text{control sample}}$. According to this formula ΔΔCt for the control group was 0 and *VEGF* gene expression in this group equaled 1. The results of *VEGF* and *EG-VEGF* gene expression for patients were expressed as mean ± S.D. The results of *VEGF* and *EG-VEGF* gene expression for patients were expressed as mean ± S.D.

Statistical analysis

Statistical analyses were performed with STATISTICA 10.0 software. A $\alpha = 0.05$ error was considered to be significant for all comparisons. The Shapiro-Wilk test was used to verify normality. All continuous variables were non-normally distributed. They were presented as median and interquartile range. Categorical variables were presented as number (percentage). $\chi 2$ tests and taper factors were used for the correlation of categorical variables. The Mann-Whitney U-test, Kruskal-Wallis and multiple repetition tests compared continuous variables and Friedman's test, whilst categorical by Fisher's test. Log-linear analyses and unconditional logistic regression were performed to adjust for categorical variables. For binominal variables were calculated adjusted Odds ratio (ORs) with 95% confidence interval (95%CIs).

The aim of the ROC curve analysis is to set the cut-off point, which denotes such an amount of the parameter of the quantitative feature, which statistically distinguishes one group of patients from the other. The ROC curve analysis was not related to individual elements. Knowing the value of the quantitative parameter of a particular patient, it allows to estimate the chance to assign the patient to one of the groups. The addition of the odd ratio analysis to the ROC curve analysis provides more accurate information about the chances of belonging to one of the groups. The VEGF/sVEGF-R1 as a quantitative parameter was checked in all patients.

RESULTS

A total number of 148 patients were recruited for this trial and underwent IVF/ICSI procedure. Forty eight women were in natural cycles. Forty nine patients were in a group where GnRH agonist was used, and 51 were in a group where GnRH antagonist was used.

When we compare all patients in a trial, OHSS occurrence was significantly correlated with higher level of sVEGF-R1 (P = 0.003) and a lower level of VEGF (P = 0.0028) in FF from large follicles (*Fig. 1* and *2*), and these observations were made regardless of the stimulation protocol.

According to ROC analysis VEGF/sVEGF-R1 ratio for patients in COH groups, above which the risk of developing OHSS is very low, was found to be 0.281 pg/ml, with OR 0.1 (95% CI 0.01 – 0.29, P = 0.0006). AUC – 0.738, P = 0.042, (95% CI 0.656 – 0.82) (*Fig. 3*).

There were no statistical differences between the three groups in mRNA levels of VEGF and EG-VEGF in GC on a day of oocyte retrieval from small (respectively P = 0.28 and P = 0.39) and large follicles (respectively P = 0.42 and P = 0.94).

When we compared both COH protocols with control group, we found statistically higher levels of sVEGF-R1 in FF

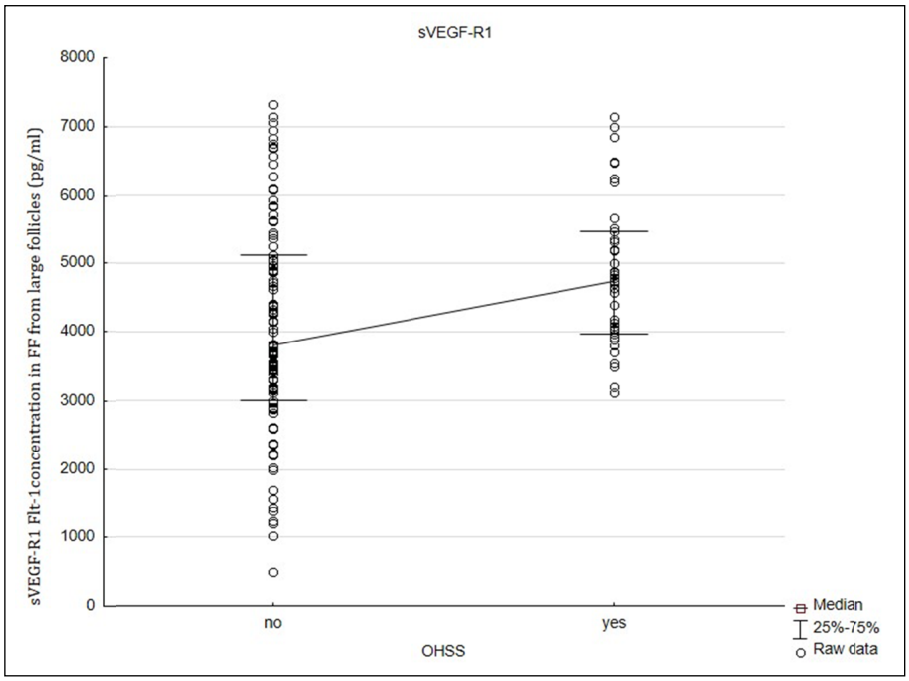

Fig. 1. sVEGF-R1 Flt-1concentration in FF from large follicles (pg/ml).

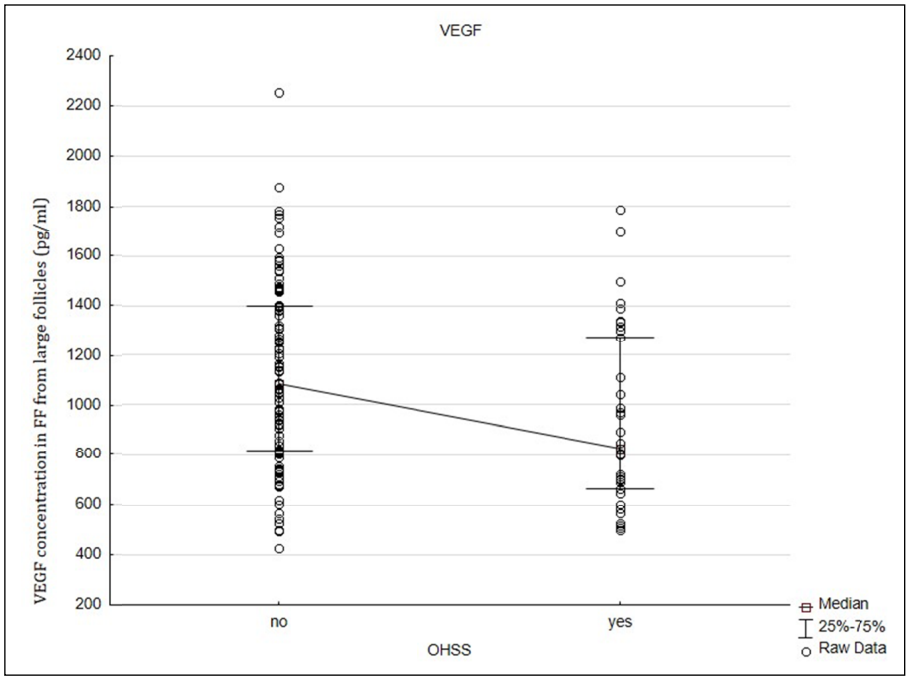

Fig. 2. VEGF concentration in FF from large follicles (pg/ml).

from large follicles (control versus long protocol group: 2763.43 ± 1072.85 pg/ml versus 4965.06 ± 1124.0 pg/ml, P < 0.001 and control versus short protocol group: 2763.43 ± 1072.85 pg/ml versus 4834.92 ± 1187.42 pg/ml, P < 0.001) and lower levels of VEGF in both COH groups (control versus long protocol group: 1205.83±378.3 pg/ml versus 1032.24 ± 379.69 pg/ml, P < 0.027, control versus short protocol group: 1205.83 ± 378.3pg/ml versus 998.0 ± 338.18 pg/ml, P < 0.008) (*Table 2*). In both COH groups higher level of EG-VEGF in comparison to control group was observed (control versus long protocol group: 2965.88 ± 1402.08 pg/ml versus 3625.14 ± 784.51 pg/ml, P = 0.001, control versus short protocol group: 2965.88 ± 1402.08pg/ml versus 3661.86 ± 807.29 pg/ml, P = 0.001). In the antagonist group significantly higher level of serum VEGF on a day of oocyte retrieval was also noticed compared to control group (107.75 ± 50.90 pg/ml versus 131.16 ± 65.96 pg/ml, P = 0.027) with no statistical differences between COH groups (P = 0.601).

There were no statistical differences between groups after COH in the incidence of OHSS (P = 0.8) (*Table 3*).

DISCUSSION

The results of our study showed that the VEGF/sVEGF-R1 ratio in FF on the day of oocyte retrieval in women undergoing

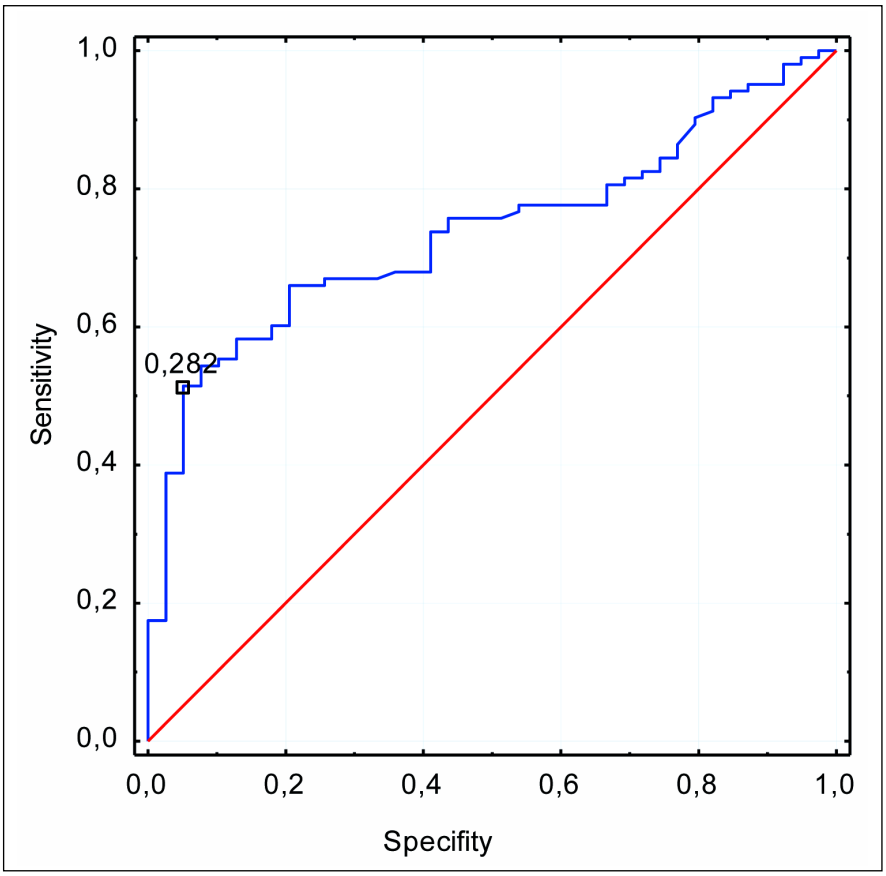

Fig. 3. ROC curve for OHSS versus VEGF/sVEGF-R1/sFlt1 in FF on a day of oocyte retrieval.

IVF procedure, regardless of the type of stimulation protocol, might predict the risk of developing OHSS. To the best of our knowledge this is the first paper in the literature to show interplay among VEGF, EG-VEGF and sVEGF-R1 and the correlation between their concentration and OHSS risk.

A number of articles have shown connection between the amount of VEGF and the presence of OHSS in patients undergoing IVF procedure. In support of this theory there are studies that indicate increased levels of VEGF in serum, FF and peritoneal fluid in women who develop OHSS (7, 22, 23), and higher serum level of VEGF in women at risk of OHSS, which correlated with clinical course of the syndrome (24). While the studies summarized above indicate a link between VEGF levels and the development of OHSS, others do not (25-28). We propose that there are several reasons behind these discrepancies. Firstly, it might be a consequence of not homogenous groups in trials or not proper selection of patients for COH protocol. Secondly and most importantly, overwhelming number of authors compare patients in selected COH protocol to patients in another COH protocol, or prepare the papers based on results from patients within one COH protocol. Thirdly, a small number of patients in a trial might influence the results. The fourth factor that might influence final conclusions is that the level of VEGF is measured in one patient in serum, while in the other one in plasma. Also important to mentioned is a fact that the VEGF gene is highly polymorphic and in some way this polymorphism may influence the results of all published papers. There are more than 80 SNPs in this region of the human genome. Some of these SNPs, which are localized in the promoter region of VEGF genes (VEGF G-405C and VEGF C-2578A) may have an impact on VEGF production in peripheral blood and in other tissues. The potential role of VEGF SNPs was evaluated in terms of spontaneous abortion, recurrent pregnancy loss, preterm labour, pre-eclampsia or gestational diabetes (29).

Soluble receptors contribute to the amount of free, biological active VEGF in FF and later in serum by binding VEGF and thereby depleting the amount of free circulating biological active VEGF. sVEGF-R1 plays a role of modulator of available VEGF in FF and serum of patients with OHSS (10, 11). VEGF upregulates sVEGF-R1 expression in human vascular endothelial cells (12). The VEGF induced sVEGF-R1 upregulation can operate as a negative feedback system (30). During COH increasing level of VEGF induces increased sVEGF-R1 concentration, which in a later process in the ovary will be combined with VEGF making the free VEGF less available for VEGFR in endothelium. According to Gruemmer, some - not precisely known - proteins, produced and secreted by GC, which are contained in FF, inhibit sVEGF-R1 production, and this regulation also occurs on a posttranscriptional level (31).

Previous studies indicate that there are many regulatory systems involved in regulation of reproductive activity (32). It is possible that some women have a better intrinsic ability to neutralize the effects of the VEGF than others through higher sVEGF-R1 concentration in FF. This ability would, therefore, explain why comparable degrees of stimulation do not result in comparable risk of OHSS (33).

Based on our results we hypothesized that in early stages of OHSS (level I and II of OHSS according to Golan classification), when an ovary works in a physiological manner and responds to COH, the level of sVEGF-R1 grows as the level of free and not bound VEGF in follicular fluid decreases, blocking it and inhibiting the development of OHSS. When this

Table 2. VEGF and sVEGF-R1 concentration.

	Control (n = 48)	**GnRH agonist** (n = 49)	**GnRH antagonist** (n=51)	**P-value control vs. GnRH agonist**	**P-value control vs. GnRH antagonist**	**P-value GnRH agonist vs. GnRH antagonist**
VEGF in serum (pg/ml ± S.D.)	107.75±50.90	125.98±66.88	131.16±65.96	P=0.07	P=0.03*	P=0.6
VEGF in FF (pg/ml±S.D.)	1205.83±378.3	1032.24±379.69	998.0±338.18	P<0.027*	P<0.008*	P=0.8
sVEGF-R1 in serum (pg/ml±S.D.)	151.44±61.33	126.0±34.61	137.0±33.18	P=0.07	P=0.6	P<0.027*
sVEGF-R1 in FF (pg/ml±S.D.)	2763.43±1072.85	4965.06±1124.0	4834.92±1187.42	P<0.001*	P<0.001*	P=0.5

*- statistical significance

Table 3. OHSS incidence among groups.

	Control (n = 48)	**GnRH agonist** (n = 49)	**GnRH antagonist** (n = 51)	**P-value control vs. GnRH agonist**	**P-value control vs. GnRH antagonist**	**P-value GnRH agonist vs. GnRH antagonist**
OHSS (n/%)	0	12/24.48	16/31.37	P < 0.001*	P < 0.001*	P = 0.22
I level	0	9/75	13/81.2	P < 0.001*	P < 0.001*	P = 0.35
II level	0	3/25	2/12.5	P < 0.001*	P < 0.001*	P = 0.21
III level	0	0/0	1/6.25	1	P = 0.04*	P = 0.19

*- statistical significance

mechanism fails, high levels of VEGF and small levels of sVEGF-R1 are found also in serum, and full clinical presentation of OHSS is observed. As mentioned above, most of our patients were in the early stages of OHSS and this was why we probably did not see high levels of VEGF in serum as they were seen in papers of others authors. Blocking receptors which are soluble or not, through higher levels of ligand protein is well known biologic mechanism in ovaries and other tissues (34). It was also previously postulated that a constant VEGF/sVEGFR serum ratio might prevent adverse effects of VEGF in patients under long protocol in IVF/ICSI procedure (10). In our trial we can precisely set up a cut-off point based on VEGF/sVEGF-R1 concentration ratio in FF on a day of oocyte retrieval (0.281) for patients in COH groups, above which the risk of developing OHSS is very low, with OR 0.1 (95% CI 0.01 – 0.29, P = 0.0006) (*Fig. 3*). High levels of sVEGF-R1 in FF may decrease free (unbound) VEGF concentration in FF and additionally in serum, and therefore protect against OHSS. Growing level of sVEGF-R1, which lowers the level of free VEGF in FF on a day of oocyte retrieval after COH, is a consequence of a natural mechanism of ovary homeostasis. When this mechanism is impaired or the level of VEGF is too high after stimulation, a patient will develop OHSS.

Lower serum levels of sVEGF-R2 are associated with the occurrence of OHSS and the severity of the disease increases with the decrease in sVEGF-R2 serum levels, but not with the increase in the amount of VEGF (35). In another study plasma sVEGF-R1 levels was lower only in the severe form of early-onset OHSS, but in the mild form of early-onset OHSS it was higher (36).

It is noteworthy that both papers - contrary to our trial - showed the results after a few days of oocyte retrieval and the results were based only on serum or plasma levels, not on FF concentration. In a paper by Pau, the stimulation protocol was not defined, whereas in Pietrowski's paper included a short protocol with GnRH antagonist. The sample size was also small, which might influence the results. In our trial we analyzed FF and serum on a day of oocyte retrieval from both protocols, comparing one to another and to the control group, which seems to be more reliable to define patomechanism of OHSS as far as the ovary is concerned as the primary organ of its origin. In all our patients we found opposite correlation between sVEGF-R1 levels in FF from large follicles and its level in the serum (high level of sVEGF-R1 in FF, small level in serum, P = 0.01), and there was a correlation between sVEGF-R1 levels in large and small follicles (P = 0.01).

In the literature there is only one paper presenting FF and serum levels of EG-VEGF in patient during COH, with GnRH agonist protocol, and it was compared with OHSS risk (37). It showed that EG-VEGF and VEGF levels were much higher in FF than in serum. They also found that FF level of EG-VEGF is positively correlated with FF level of VEGF, suggesting that their functions act synergistically in follicle development.

We also hypothesize that VEGF and EG-VEGF act synergistically in causing OHSS, with EG-VEGF being an important factor as an ovary-specific vasoactive substance. EG-VEGF is not the sole component involved in a pathophysiology of OHSS. In all groups we observed linear correlation of EG-VEGF levels in large and small follicles and in the serum (P = 0.007 and P = 0.019, respectively).

sVEGF-R1 does not interact with EG-VEGF and even insignificant elevation of EG-VEGF level is likely to result in EG-VEGF acting synergistically with unbound VEGF and in OHSS development.

It was also postulated that EG-VEGF in FF might be a useful predictor of OHSS (37). When we look at the level of EG-VEGF in FF from large follicles and serum in all patients in a trial, it does not correlate with OHSS (P = 0.70 and P = 0.52, respectively). In our opinion EG-VEGF is necessary for developing OHSS by amplifying the VEGF effect, especially when a part of free VEGF is bound to sVEGF-Rs.

In our trial we did not observe statistical differences between groups in the incidence of OHSS after COH (long protocol group versus short protocol group 24.48% versus 31.37%, P = 0.22).

Altogether, the results of our study may trigger a new approach to the patomechanism of OHSS. This study is the first paper in literature, which shows possible interplay among VEGF, EG-VEGF and sVEGF-R1 and the correlation of their concentration with OHSS risk. It is also one of the few papers, which compare the results in patients undergoing COH protocols to patients in a natural IVF/ICSI protocol without stimulation. When we compare our study to other papers in literature, the sample sizes of our groups are impressive, but might be too small for definite conclusions. In our opinion there is a huge need to design a controlled prospective trial of an adequate sample size to determine the role of VEGF, EG-VEGF and its receptors in FF and serum in OHSS pathophysiology. We are aware that the problem is to select homogenous groups of patients to conduct such a study.

All in all, the systemic effect of VEGF, EG-VEGF and sVEGF R1 is influenced by ligand receptor protein binding interactions, and these relationships may exhibit dynamic changes during COH cycles and could contribute to the development of OHSS.

Abbreviations: COH, controlled ovarian hyperstimulation; EG-VEGF, endocrine gland-derived vascular endothelial growth factor; FF, follicular fluid; GC, granulosa cells; ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; OHSS, ovarian hyperstimulation syndrome; sVEGF-R1-Flt1, soluble receptor for vascular endothelial growth factor; VEGF, vascular endothelial growth factor

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REFERENCES

1. Alper MM, Smith LP, Sills ES. Ovarian hyperstimulation syndrome: current views on pathophysiology, risk factors, prevention, and management. *J Exp Clin Assist Reprod* 2009; 6: 3.
2. Madill JJ, Mullen NB, Harrison BP. Ovarian hyperstimulation syndrome: a potentially fatal complication of early pregnancy. *J Emerg Med* 2008; 35: 283-286.
3. Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF. Vascular permeability, vascular hyperpermeability and angiogenesis. *Angiogenesis* 2008; 11: 109-119.
4. Elchalal U, Schenker JG. The pathophysiology of ovarian hyperstimulation syndrome - views and ideas. *Hum Reprod* 1997; 12: 1129-1137.
5. Levin ER, Rosen GF, Cassidenti DL, *et al*. Role of vascular endothelial cell growth factor in ovarian hyperstimulation syndrome. *J Clin Invest* 1998; 102: 1978-1985.
6. Ajonuma LC. Is vascular endothelial growth factor (VEGF) the main mediator in ovarian hyperstimulation syndrome (OHSS)? *Med Hypotheses* 2008; 70: 1174-1178.
7. Agrawal R, Tan SL, Wild S, *et al*. Serum vascular endothelial growth factor concentrations in in vitro fertilization cycles predict the risk of ovarian hyperstimulation syndrome. *Fertil Steril* 1999; 71: 287-293.
8. Manau D, Fabregues F, Penarrubia J, Creus M, Carmona F, *et al*. Vascular endothelial growth factor levels in serum and plasma from patients undergoing controlled ovarian hyperstimulation for IVF. *Hum Reprod* 2007; 22: 669-675.
9. Mathur R, Hayman G, Bansal A, Jenkins J. Serum vascular endothelial growth factor levels are poorly predictive of subsequent ovarian hyperstimulation syndrome in highly responsive women undergoing assisted conception. *Fertil Steril* 2002; 78: 1154-1158.
10. Gutman G, Barak V, Maslovitz S, Amit A, Lessing JB, Geva E. Regulation of vascular endothelial growth factor-A and its soluble receptor sFlt-1 by luteinizing hormone in vivo: implication for ovarian follicle angiogenesis. *Fertil Steril* 2008; 89: 922-926.
11. Sela S, Natanson-Yaron S, Zcharia E, Vlodavsky I, Yagel S, Keshet E. Local retention versus systemic release of soluble VEGF receptor-1 are mediated by heparin-binding and regulated by heparanase. *Circ Res* 2011; 108: 1063-1070.
12. Hornig C, Barleon B, Ahmad S, Vuorela P, Ahmed A, Weich HA. Release and complex formation of soluble VEGFR-1 from endothelial cells and biological fluids. *Lab Invest* 2000; 80: 443-454.
13. LeCouter J, Kowalski J, Foster J, *et al*. Identification of an angiogenic mitogen selective for endocrine gland endothelium. *Nature* 2001; 412: 877-884.
14. Su MT, Lin SH, Lee IW, *et al*. Polymorphisms of endocrine gland-derived vascular endothelial growth factor gene and its receptor genes are associated with recurrent pregnancy loss. *Hum Reprod* 2010; 25: 2923-2930.
15. Lin R, LeCouter J, Kowalski J, Ferrara N. Characterization of endocrine gland-derived vascular endothelial growth factor signaling in adrenal cortex capillary endothelial cells. *J Biol Chem* 2002; 277: 8724-8729.
16. Ferrara N, LeCouter J, Lin R, Peale F. EG-VEGF and Bv8: a novel family of tissue-restricted angiogenic factors. *Biochim Biophys Acta* 2004; 1654: 69-78.
17. LeCouter J, Lin R, Ferrara N. Endocrine gland-derived VEGF and the emerging hypothesis of organ-specific regulation of angiogenesis. *Nat Med* 2002; 8: 913-917.
18. Battersby S, Critchley HO, Morgan K, Millar RP, Jabbour HN. Expression and regulation of the prokineticins (endocrine gland-derived vascular endothelial growth factor and Bv8) and their receptors in the human endometrium across the menstrual cycle. *J Clin Endocrinol Metab* 2004; 89: 2463-2469.
19. Fraser HM, Bell J, Wilson H, *et al*. Localization and quantification of cyclic changes in the expression of endocrine gland vascular endothelial growth factor in the human corpus luteum. *J Clin Endocrinol Metab* 2005; 90: 427-434.
20. Lainas TG, Sfontouris IA, Zorzovilis IZ, *et al*. Flexible GnRH antagonist protocol versus GnRH agonist long protocol in patients with polycystic ovary syndrome treated for IVF: a prospective randomised controlled trial (RCT). *Hum Reprod* 2010; 25: 683-689.
21. Golan A, Weissman A. Symposium: Update on prediction and management of OHSS. A modern classification of OHSS. *Reprod Biomed Online* 2009; 19: 28-32.
22. Krasnow JS, Berga SL, Guzick DS, Zeleznik AJ, Yeo KT. Vascular permeability factor and vascular endothelial growth factor in ovarian hyperstimulation syndrome: a preliminary report. *Fertil Steril* 1996; 65: 552-555.
23. Lee A, Christenson LK, Stouffer RL, Burry KA, Patton PE. Vascular endothelial growth factor levels in serum and follicular fluid of patients undergoing in vitro fertilization. *Fertil Steril* 1997; 68: 305-311.
24. Abramov Y, Barak V, Nisman B, Schenker JG. Vascular endothelial growth factor plasma levels correlate to the clinical picture in severe ovarian hyperstimulation syndrome. *Fertil Steril* 1997; 67: 261-265.
25. Kobayashi H, Okada Y, Asahina T, Gotoh J, Terao T. The kallikrein-kinin system, but not vascular endothelial growth factor, plays a role in the increased vascular permeability associated with ovarian hyperstimulation syndrome. *J Mol Endocrinol* 1998; 20: 363-374.

26. Chen CD, Chen HF, Lu HF, Chen SU, Ho HN, Yang YS. Value of serum and follicular fluid cytokine profile in the prediction of moderate to severe ovarian hyperstimulation syndrome. *Hum Reprod* 2000; 15: 1037-1042.
27. Enskog A, Nilsson L, Brannstrom M. Plasma levels of free vascular endothelial growth factor(165) (VEGF(165)) are not elevated during gonadotropin stimulation in in vitro fertilization (IVF) patients developing ovarian hyperstimulation syndrome (OHSS): results of a prospective cohort study with matched controls. *Eur J Obstet Gynecol Reprod Biol* 2001; 96: 196-201.
28. Ludwig M, Bauer O, Lopens A, Jelkmann W, Diedrich K. Serum concentration of vascular endothelial growth factor cannot predict the course of severe ovarian hyperstimulation syndrome. *Hum Reprod* 1998; 13: 30-32.
29. Iciek R, Wender-Ozegowska E, Mikolajczak P, Seremak-Mrozikiewicz A, Zawiejska A, Mrozikiewicz PM, *et al*. Placental vascular endothelial growth factor expression in pregnancies complicated by type 1 diabetes. *J Physiol Pharmacol* 2014; 65: 577-583.
30. Saito T, Takeda N, Amiya E, *et al*. VEGF-A induces its negative regulator, soluble form of VEGFR-1, by modulating its alternative splicing. *FEBS Lett* 2013; 587: 2179-2185.
31. Gruemmer R, Motejlek K, Berghaus D, Weich HA, Neulen J. Regulation of soluble vascular endothelial growth factor receptor (sFlt-1/sVEGFR-1) expression and release in endothelial cells by human follicular fluid and granulosa cells. *Reprod Biol Endocrinol* 2005; 3: 57.
32. Romerowicz-Misielak M, Tabecka-Lonczynska A, Koziol K, *et al*. Changes in gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor gene expression after an increase in carbon monoxide concentration in the cavernous sinus of male wild boar and pig crossbread. *J Physiol Pharmacol* 2016; 67: 431-442.
33. McElhinney B, Ardill J, Caldwell C, Lloyd F, McClure N. Ovarian hyperstimulation syndrome and assisted reproductive technologies: why some and not others? *Hum Reprod* 2002; 17: 1548-1553.
34. Gregoraszczuk EL, Rak A. Superactive human leptin antagonist reverses leptin-induced excessive progesterone and testosterone secretion in porcine ovarian follicles by blocking leptin receptors. *J Physiol Pharmacol* 2015; 66: 39-46.
35. Pietrowski D, Szabo L, Sator M, Just A, Egarter C. Ovarian hyperstimulation syndrome is correlated with a reduction of soluble VEGF receptor protein level and a higher amount of VEGF-A. *Hum Reprod* 2012; 27: 196-199.
36. Pau E, Alonso-Muriel I, Gomez R, *et al*. Plasma levels of soluble vascular endothelial growth factor receptor-1 may determine the onset of early and late ovarian hyperstimulation syndrome. *Hum Reprod* 2006; 21: 1453-1460.
37. Gao MZ, Zhao XM, Sun ZG, Hong Y, Zhao LW, Zhang HQ. Endocrine gland-derived vascular endothelial growth factor concentrations in follicular fluid and serum may predict ovarian hyperstimulation syndrome in women undergoing controlled ovarian hyperstimulation. *Fertil Steril* 2011; 95: 673-678.

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