E-selectin, resistin and reactive oxygen species levels in GnRH -agonist and -antagonist protocols in IVF/ICSI: a prospective cohort study

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Received: 11 January 2015 / Accepted: 22 April 2015 / Published online: 1 May 2015
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Abstract

Purpose To compare E-selectin, resistin and reactive oxygen species (ROS) levels in serum and follicular fluid (FF) of subfertile women undergoing Controlled Ovarian Hyperstimulation (COH) during IVF/ICSI cycles, using GnRH-agonist and -antagonist protocols.

Methods In this prospective cohort study, 85 subfertile women undergoing IVF/ICSI were included. Participants underwent the GnRH-agonist and -antagonist protocols; and blood samples were collected at three time points: basic (at start of COH), on the day of hCG and at oocyte retrieval (OR); and from the FF from the first follicle aspirate. Clinical and IVF cycle characteristics, were compared between groups, together with the levels of E-selectin, resistin and ROS in

serum and FF, through ELISA. Their prognostic value on pregnancy outcomes was examined.

Result(s) Examining molecules levels are increasing in serum, from start of COH until OR, irrespectively of the protocol used; FF levels at OR were similar to those in serum at that day. Resistin FF levels were lower in GnRH agonists, compared with the antagonist protocol. Resistin levels at start of COH were associated with clinical pregnancy rates, and this remained significant following adjustment for age, BMI and IVF protocol used, while values of >13.5 ng/ml were associated with a six times greater odd of a pregnancy.

Conclusion E-selectin, resistin and ROS levels are increasing during COH, reaching their highest values at OR, with comparable values measured in the FF at that time. Resistin values >13.5 ng/ml are linked with a 6-fold increase on the odds of a pregnancy.

Capsule This prospective cohort study showed that E-selectin, resistin and ROS levels are increasing during controlled ovarian hyperstimulation for IVF/ICSI, reaching their highest values at the day of oocyte recovery, with comparable values measured in the follicular fluid at that time. Resistin values >13.5 ng/ml are linked with a 6-fold increase on the odds of a pregnancy.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-015-0489-z) contains supplementary material, which is available to authorized users.

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Keywords E-selectin · Resistin · Reactive oxygen species · IVF · ART

Introduction

Selectins are glycoproteins that consist of a group of endothelial cell adhesion molecules, mediating the recruitment and adhesion of circulating leukocytes towards, among other targets, inflamed tissues. Their expression is induced by pro-inflammatory cytokines and through their activity during various states of inflammation/infection and endothelial activation, they serve as potential biomarkers. Their role in female reproduction has already been established, as they are involved in the regulation of the ovarian function, menopause and the pathogenesis of preeclampsia [1]. The family includes 3 types, namely L-, P-, and E-selectin. L-selectin, is expressed by lymphocytes and neutrophils and regulates their

transmigration to endothelial cells at the inflammation area, exerting a facilitating role in embryo implantation [2]; its expression and circulating levels are fluctuating during controlled ovarian hyperstimulation [COH], from the initiation of ovarian stimulation until the implantation window [3, 4]. P-selectin, is stored in platelets and endothelial cells and mediates the attachment of neutrophils, T-lymphocytes and monocytes to the endothelial cells; its levels are increased during COH, but are unrelated to steroid levels or the amount of gonadotrophins used [5]. E-selectin, is expressed and produced exclusively by stimulated vascular endothelia and guides the memory cells to the inflammation area. It can be detected in endometritis cases, but also in follicular aspirates during IVF in human ovarian micro-vascular endothelium [6–8]; its levels have been correlated with the clinical presentation and biological aspects of ovarian hyperstimulation syndrome [OHSS] [1, 9] and the differentiated hormonal states induced by the administration of GnRH agonists [10] or oral estrogens [11].

Resistin is a 12.5 kDa cysteine-rich protein, secreted by adipocytes that functionally impairs glucose metabolism and induces insulin resistance [12]; it has also been associated with proinflammatory changes in the vascular endothelium [13], pathways linked to various inflammatory diseases [14] and angiogenesis [15]. In humans, resistin is mainly produced by macrophages [16]. Animal studies have demonstrated a contributory function in testosterone production and pubertal hypothalamic–pituitary signaling, attributing a role as an endocrine mediator of the reproductive axis [14, 17, 18] and a potential one concerning obesity-associated insulin resistance [12, 19, 20]. In reproduction, there has been a controversy on its possible linkage with anovulatory PCOS [21–23], or non-PCOS women undergoing IVF [24], further negating the impact of resistin on pregnancy outcome parameters [25, 26]. Recent studies have demonstrated the presence of resistin in human granulosa [27], as well as in cumulus, theca cells and oocytes [28]. Also, possible feedbacks through LH and progesterone [positive] and E2 [negative] have been postulated [14]. In biological terms, resistin reduces the secretion of both P450scc and P450 aromatase protein levels, and IGF-IR signaling in response to IGF-I in granulosa cells [28].

In the female reproductive system and embryo development, Reactive Oxygen Species (ROS) are believed to play an important role, since they are involved in ovarian steroidogenesis and ovulation, oocyte maturation, embryo compaction and blastocyst development and implantation, along with luteolysis and luteal maintenance during pregnancy [29, 30]. Although there are reports favoring a direct link between ROS levels and various types of subfertility, this association has not been established and elucidated, as yet [31].

The underlying mechanism of the association between E-selectin and resistin and COH during IVF treatment, is attributed to the link of inflammation and autoimmune response

with COH [32]; both molecules are expressed on human granulosa cells. Concerning ROS levels, an optimum level of ROS is necessary to guarantee healthy oocyte development, opposing to the notable imbalance in the ovarian environment after COH, with excessively high levels to exert a detrimental effect due to oxidative stress [33–36].

We performed a prospective study to compare E-selectin, resistin and ROS levels at different time points in serum and follicular fluid of subfertile women undergoing COH as part of an IVF treatment course, using both GnRH agonist and antagonist protocols. During the study we also assessed possible associations of these molecules with the reproductive outcome.

Materials and methods

Patient population and study design

This is a single center, prospective, cohort study performed at the Assisted Reproductive Unit of the 3rd Department of Obstetrics and Gynecology of the Athens University School of Medicine, “Attikon” hospital, from September 2011 to December 2013. The study was approved by the Scientific Board (protocol no 187/24-06-08) and Bioethics Committee of the hospital (no of approval 6/07-07-08). An informed consent was obtained from all patients included in this study.

Subjects

Eighty-five cycles of IVF in 85 women with primary or secondary subfertility were analyzed. The initial number of participants recruited was 95, but sufficient data for analysis was obtained for 85 women: seven of them withdrew their consent denying the blood sampling, two of them were excluded due to high risk of developing ovarian hyperstimulation syndrome and one due to poor ovarian response to stimulation. Subfertility factors were well recorded through the typical processes of the Unit, categorized as follows: female (tubal infertility, ovulatory dysfunction and endometriosis), male factor and unexplained infertility. Exclusion criteria for the participation in the study were: age >42 years old, basal hormonal levels of FSH at day 3 of the menstrual cycle [>15 IU/L], other protocols towards oocyte retrieval (natural or mild IVF cycles). In addition, women with known previous poor ovarian response to ovarian stimulation were excluded.

Patients were enrolled during a 2 years period and participated in the study only once. The study population was classified into two groups, according to the protocol used for pituitary down-regulation (GnRH-agonist and GnRH-antagonist group). Treatment allocation was achieved according to the number of the file of each patient (0 to 3 vs. 4 to 9 as a last digit), respectively. In order to avoid potential sources of

bias, we included participants who were referred to the Unit consecutively, and the number selected was based on previous reports [24–26]. The enrollment of the participants to the two protocols was based on the standard clinical practice of the Unit.

Stimulation protocol and IVF

For the GnRH-agonist protocol, Triptorelin [Gonapeptyl, 0.1 mg (Ferring Pharmaceutical Hellas A.E.) or Arvekap, 0.1 mg (Ipsen, EPE)] was administered subcutaneously daily during the midluteal phase or the second day of the menstrual cycle.

For the GnRH antagonist protocol, ovarian stimulation began on the second day of the cycle and the antagonist, either Cetrorelix (Merck Serono Europe Limited, UK) or Orgalutran (Merck Sharp & Dohme Limited, UK) was initiated as soon as the leading follicle reached a diameter of 14 mm. In anovulatory patients, triptorelin was administered the day after completion of a course of oral contraceptive pills. Once pituitary down-regulation and ovarian suppression have been achieved, ovarian stimulation with exogenous gonadotropins was started while GnRH agonist administration was continued concomitantly until the day of human chorionic gonadotrophin [hCG] administration. Recombinant FSH in the form of either follitropin alpha (Gonal-F; Merck Serono Europe Ltd) or follitropin beta (Puregon Merck Sharp & Dohme Ltd) was administered subcutaneously.

For cycle monitoring, transvaginal ultrasonography every 2–4 days (or as required) was performed, whereas supplemental estradiol monitoring was conducted for PCOS patients and those at high risk of OHSS. Starting doses were adjusted individually according to the age, FSH, AMH levels and previous response to IVF cycles of each participant, while further adjustments and monitoring frequency were dependent upon participants' response to stimulation. When two or more follicles reached a diameter of 18 mm, human chorionic gonadotrophin [10,000 IU Pregnyl (N. V. Organon, Netherlands) or 250mcg Ovitrelle (Merck Serono Europe Ltd, Germany)] was administered prior to transvaginal ultrasound-guided oocyte retrieval (OR) 36–38 h later. Intracytoplasmic sperm injection (ICSI) was performed both in cases of male subfertility, and in assisted reproductive history with recurrent implantation failure and low fertilization rate. Depending on the embryo quality, embryo transfer (ET) was performed either 2 or 3 days after the OR. Luteal phase support was achieved by transvaginal administration of progesterone in the form of either Utrogestan vaginal suppositories (Angelini Pharma Hellas A.B.E.E.) or Vasclor gel (Verisfield; U.K. Ltd) or a combination of both.

Clinical pregnancy was confirmed by a transvaginal ultrasound scan at 7 weeks of gestation, suggested after a positive β -hCG 14 days after ET.

Samples collection

Blood samples were taken from both groups of participants at a specially designed room in the Unit, from the medical and/or nursing staff. Follicular fluid [FF] was collected from the first follicle aspirate [$\Phi \geq 1.8$ cm] and centrifuged at 1000 rpm for 10 min immediately, then frozen for storage at -60 °C. Blood samples were collected at three time points [TP]: basic [on the day before the ovarian stimulation on both groups] [TP1], between 8:00 and 10:00 am on the day of hCG administration [TP2] and 5 min before the OR [TP3]. All samples were kept at room temperature for at least 30 min to allow coagulation and were then centrifuged at 3500 rpm [revolutions per minute] for 10 min in order to isolate the serum and to remove cellular components from the FF. The clear supernatant was frozen in -60 °C until it was assayed.

Sample detection methods

Serum levels of E-selectin, resistin and ROS were measured by using a commercially available monoclonal antibody-based sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer guidelines (Raybiotech, USA). A monoclonal antibody specific for resistin or E-selectin was pre-coated on the microplate, whereas the lowest detectable levels were 1.4 and 24.69 ng/ml, for the two molecules, respectively.

Preparation of standards

A Lyophilised Standard for the molecules was diluted in a 400 μ l Assay Diluent A (30 ml of animal serum with 0.9 % sodium azide, as preservative) to prepare a 50 ng/ml concentration. Four μ l were added in a tube labeled as A, which already contained 496 μ l of Assay Diluent. Six more tubes were further prepared, with 300 μ l of Assay Diluent A into each. From tube A, 200 μ l were extracted and put into tube B, from tube B another 200 μ l and placed into tube C, and the procedure was continued up to the last tube (seventh), in order to obtain a Standard Curve of 0 to 40 ng/ml for Resistin, 0 to 180 ng/ml for E-selectin and 0 to 10 ng/ml for ROS.

A 100 μ l of the Lyophilised Standard was added in double and put into each well of a 96 well plate. Wells were covered and incubated for 2.5 h at room temperature with gentle shaking. After the incubation, the solution was discarded and washed four times with Wash solution. The washing was achieved by filling each well with Wash Buffer [300 μ l] using a multi-channel pipette. The complete removal of the liquid at each step was essential for obtaining a good performance. After the last wash, any remaining Wash Buffer was removed by inverting the plate against paper towels. A 100 μ l of the prepared antibody (Biotin -antibody for ROS / Biotinylated Serum for E- Selectin / Resistin) was added to each well and

incubated for 1 h at room temperature with gentle shaking. After the incubation, the solution was discarded and the washing stage was repeated. A 100 µl of the prepared Solution (HRP -Avidin for ROS / Streptavidin for E- Selectin / Resistin) was then added to each well and incubated for 30 min at room temperature with gentle shaking. After the incubation, the solution was discarded and the washing stage was repeated. A 50 µl of TMB (Substrate incubate) solution was then added to each well and incubated for 30 min at room temperature in the dark. A 50 µl of Stop Solution (used for all molecules) was then added to each well and the plate was read immediately at 450 nm, using an ELISA micro-plate reader: absorbance values were read and printed.

Calculation of results - comparisons

The mean absorbance was calculated for each set of duplicate standards and samples. A Standard curve on log-log graph paper was plotted, placing the standard concentrations on the x-axis and absorbance values on the y-axis. The best-fit straight line was drawn through the standard points.

Comparisons were performed between the two study groups with respect to demographics, clinical and IVF cycle characteristics, day-3 embryo quality and clinical pregnancy rates. E-selectin, resistin and ROS levels were measured at three time points in maternal serum and FF, followed by a direct comparison on the protein levels according to the protocol used. In addition, we examined whether the respective protein levels could contribute any prognostic value on pregnancy rates and on this basis, our intention was to propose possible cut-off values following data analysis. Adjustments in terms of age, BMI and IVF protocol were also performed.

Statistical methods

Categorical variables were expressed as percentages of the corresponding population and were compared using the χ^2 test. Continuous variables were expressed as mean $\pm$ SD and compared using the Student *t*-test for independent or paired samples accordingly, or the Mann–Whitney *U* test and Wilcoxon signed rank test, according to whether variables followed a normal distribution, as assessed by the Kolmogorov-Smirnov test. Analysis of variance [ANOVA] for repeated measures with IVF protocol as a covariate [2-way ANOVA] was used to assess the effect of protocol on E-selectin, resistin and ROS levels. Results were provided by the Sphericity test or the Greenhouse-Geisser test in case the criteria for sphericity were not met. Bonferroni correction was performed to adjust for multiple comparisons testing. Univariable and multivariable logistic regression analysis was used to estimate the Odds Ratios (OR) of E-selectin, resistin and ROS levels on the likelihood of pregnancy. Cut-off analysis using ROC curve was used to identify the optimal

cut-off value, which maximizes the sum of sensitivity and specificity of biomarkers on the probability of pregnancy if the analysis showed significant association of the biomarkers with the outcome. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the SPSS 13.0 statistical software package (SPSS, Inc, Chicago, IL).

Results

Ninety-five women were initially enrolled, but sufficient data was available for 85, with an average age (mean $\pm$ SD) of 35.7 ± 4.9 years. Regarding the stimulation protocol, 33 women ($n=33$, 38.8 %) were treated with the GnRH-agonist protocol, whereas 52 were treated with the GnRH-antagonist ($n=52$, 61.2 %). Demographics, clinical and IVF cycle characteristics of the study population are presented in Table 1.

No significant difference was found between the two study groups, apart from the (expected) duration of days between the two protocols studied (25.4 ± 7.5 vs. 12.8 ± 1.8 , $p < 0.001$). For 37 of the participants (43.5 %) this was their first IVF cycle, whereas 48 (56.5 %) had undergone one or more IVF cycles in the past. Recombinant FSH was the drug used for ovarian stimulation in the majority of women ($n=62$, 72.9 %) and no significant differences were observed between the two groups ($p=0.300$).

No significant differences were found in terms of embryo quality per grade (1–4) (Supp. Table 1). Clinical pregnancy rate of the study population was 27.1 % ($n=23$ out of 85), whereas it was higher in the GnRH-agonist group compared with the antagonist ($n=15$, 45.5 % vs. $n=8$, 15.4 %, respectively, $p=0.002$), that is, women in the GnRH-antagonist group had 78 % less odds of pregnancy compared to women in the agonist (Odds Ratio (OR)=0.218 $\mu\epsilon$ 95 % CI: 0.079–0.604, $p=0.003$). After adjustment for age and number of previous IVF cycles, the association remained statistically significant (OR=0.232, $\mu\epsilon$ 95 %CI: 0.083–0.652, $p=0.006$).

E-selectin

Repeated measures ANOVA showed that E-selectin levels increased significantly from TP1 to TP3 for the total sample and for both protocols (all p values < 0.001), but there was no significant difference with respect to the IVF protocol used ($F=0.068$, $p=0.800$) (Table 2, Supp. Fig. 1–2). Also, FF values were significantly higher compared to those at TP1 and TP2 ($p < 0.001$ and $p=0.015$, respectively), but not at TP3 ($p > 0.05$) (Table 2).

Logistic regression analysis showed that E-selectin levels at all TP and in the FF were not associated with pregnancy rates (all p -values > 0.05).

Table 1 Demographics and clinical characteristics in the total sample and per IVF protocol

Characteristic	Total (<i>n</i> =85)	GnRH-agonists (<i>n</i> =33)	GnRH-antagonists (<i>n</i> =52)	<i>p</i> -value
Age (years)	35.7±4.9	34.7±4.5	36.3±5.1	0.141
Body Mass Index (kg/m ²)	24.2±4.3	25.0±5.2	23.7±3.6	0.166
Smokers, <i>n</i> (%)	37 (43.5 %)	15 (45.5 %)	22 (42.3 %)	0.776
Subfertility				
Primary, <i>n</i> (%)	65 (76.5 %)	28 (84.8 %)	37 (71.2 %)	0.147
Secondary, <i>n</i> (%)	20 (23.5 %)	5 (15.2 %)	15 (28.8 %)	
Male subfertility, <i>n</i> (%)	30 (35.3 %)	14 (42.4 %)	16 (30.8 %)	0.273
Female subfertility, <i>n</i> (%)	38 (44.7 %)	15 (45.5 %)	23 (44.2 %)	0.912
Tubular	16 (18.8 %)	8 (24.2 %)	8 (15.4 %)	0.309
Ovary	23 (27.1 %)	7 (21.2 %)	16 (30.8 %)	0.334
Endometrial	5 (5.9 %)	1 (3.0 %)	4 (7.0 %)	0.373
Male and female subfertility, <i>n</i> (%)	6 (6.1 %)	2 (7.7 %)	4 (7.1 %)	0.294
Unexplained subfertility, <i>n</i> (%)	23 (27.1 %)	6 (18.2 %)	17 (32.7 %)	0.142
AMH levels (ng/ml)	2.7±1.0	2.6±0.80	2.8±1.2	<i>p</i> =0.55
IVF cycle duration (days)	17.7±7.8	25.4±7.5	12.8±1.8	<0.001*
LH addition to stimulation protocol	26 (30.5 %)	11 (33.3 %)	15 (28.8 %)	0.662
Mean dosage of FSH (IU/day)	277±107	269±80	282±122	<i>p</i> =0.557
Mean number of oocytes retrieved	7.4±4.0	7.6±3.5	7.3±5.7	<i>p</i> =0.788
Clinical pregnancy rate, <i>n</i> (%)	23 (27.1)	15 (45.5)	8 (15.4)	<i>p</i> =0.002*

p*<0.05Resistin**

Repeated measures ANOVA revealed that resistin levels increased significantly from TP1 to TP3 for the total sample and for both protocols (all *p* values<0.001), but there was no

difference with respect to the IVF protocol used (*F*=1.88, *p*=0.147) (Table 2).

FF levels demonstrated a statistically significant decrease in the GnRH-agonist group as compared to the GnRH-antagonist (16.9±1.3 vs. 17.5±1.1, *p*=0.031) (Fig. 1, Supp.

Table 2 E-selectin, resistin and ROS levels in the total sample, per time-point (TP) and per IVF protocol

E-Selectin	Total	GnRH-agonists	GnRH-antagonists	<i>p</i> -value
E-Selectin TP1	35.3±5.1	36.0±4.8	34.7±5.4	0.429
E-Selectin TP2	50.0±10.0	46.5±9.6	52.6±9.6	0.067
E-Selectin TP3	63.9±11.8 [§]	64.8±9.5	63.3±13.1	0.616
E-Selectin FF	61.6±11.6 [§]	60.0±12.1	62.9±11.1	0.292
Resistin	Total	GnRH-agonists	GnRH-antagonists	<i>p</i> -value
Resistin TP1	13.1±1.7	13.7±2.1	12.7±1.3	0.065
Resistin TP2	16.1±1.2 [#]	16.1±1.8	16.1±0.9	0.931
Resistin TP3	17.7±1.3 [#]	17.8±1.4	17.7±1.3	0.769
Resistin FF	17.2±1.2 [#]	16.9±1.3	17.5±1.1	0.031*
ROS	Total	GnRH-agonists	GnRH-antagonists	<i>p</i> -value
ROS TP1	2.6±0.5	2.6±0.6	2.6±0.5	0.960
ROS TP2	4.8±0.6 [#]	4.8±0.7	4.8±0.6	0.817
ROS TP3	6.6±1.0 [#]	6.7±0.8	6.6±1.1	0.491
ROS FF	6.6±1.3 [#]	6.6±1.2	6.7±1.3	0.709

[§] compared to TP1, *p*<0.001 after Bonferroni correction for multiple comparisons

[¶] compared to TP1, *p*=0.002 after Bonferroni correction for multiple comparisons

**p*<0.05

[#] compared to baseline values (TP1), *p*<0.001 after Bonferroni correction for multiple comparisons

[#] compared to TP1, *p*<0.001 after Bonferroni correction for multiple comparisons

Fig. 1 Boxplots of E-selectin levels per IVF protocol and at the three time-points (TP) and the follicular fluid (FF)

Fig. 3). Also, they were higher compared with TP1 ($p < 0.001$), but not with TP2 and TP3 (both p -values > 0.05) (Table 2).

Univariable logistic regression analysis showed that resistin levels at TP1 were associated with clinical pregnancy rates (OR=1.781, 95 %CI=1.116–2.844, $p=0.016$). The

association remained significant after adjusting for age, BMI and IVF protocol used (OR=1.733 $\mu\epsilon$ 95 %CI=1.034–2.904, $p=0.037$). ROC analysis identified that resistin value of 13.5 ng/ml was the cut-off point that maximizes the sum of sensitivity and specificity (sensitivity=72.7 %, specificity=70.5 %) (Area under the curve (AUC)=0.739, $p=0.018$) (Fig. 2). Women with levels of resistin > 13.5 ng/ml were six times more likely to achieve pregnancy compared to women with resistin < 13.5 (OR=6.4 $\mu\epsilon$ 95 %CI=1.402–29.209, $p=0.017$). This association remained significant after adjustment for age, BMI and IVF protocol used (OR=5.592 $\mu\epsilon$ 95 %CI=1.183–26.435, $p=0.030$).

No further association was found at TP2, TP3 and in the FF (p values 0.456, 0.185, and 0.315, respectively) between resistin and pregnancy rates.

ROS

Repeated measures ANOVA revealed that ROS levels increased significantly from TP1 to TP3 for the total sample and for both protocols (all p values < 0.001 , $F=61.1$), (Supp. Fig. 4–5), but there was no difference with respect to the IVF protocol used ($F=0.83$, $p=0.485$) (Table 2).

ROS levels at all TPs demonstrated no prognostic value on pregnancy rates (all p -values > 0.05). Also, FF ROS levels were higher as compared to ROS levels at TP1 and TP2 (both p -values < 0.001), but there was no difference between FF and TP3 (p -values > 0.05) (Table 2).

Diagonal segments are produced by ties.

Fig. 2 ROC curve of resistin levels on the probability of pregnancy

Discussion

Eighty-five women were recruited in this prospective cohort study, divided into two study groups with similar, in terms of demographics, clinical and IVF cycle characteristics. We evaluated the levels of E-selectin, resistin and ROS in both serum and FF in sub-fertile women undergoing COH during IVF, comparing GnRH-agonist and -antagonist protocols for down-regulation.

The levels of the studied molecules were increased during COH, from day 1 of ovarian stimulation until oocyte retrieval (OR), irrespectively of the protocol used, with higher levels detected in the FF compared to serum, except from the day of OR, where levels were lower. Resistin levels were lower in the FF of women receiving GnRH agonists, compared with the antagonist protocol. Interestingly, resistin levels before the initiation of COH were associated with clinical pregnancy rates, and this remained significant following adjustment for age, BMI and IVF protocol used. Moreover, ROC analysis identified that values of >13.5 ng/ml were associated with a 6-fold increase in the odds of a clinical pregnancy.

E-selectin and ROS serum levels were increased from day 1 of COH, reaching a peak on the day of OR, with values comparable to those detected in FF. These differences were found to be unrelated to the IVF protocol used. Both appear to respond positively to the hormonal rise during COH, without being affected by pituitary suppression. Importantly, none of the values measured for E-selectin and ROS has been associated with the likelihood of pregnancy. Of note, concerning ROS, we have found no similar studies during our literature search in order to perform a direct comparison with our reported data. As for E-selectin, Orvieto et al. [3], studying 14 patients undergoing IVF, reported similar results, finding significantly higher levels on the day of OR compared with the days of COH commencement and HCG (but with no difference between the two); in addition authors spotted no correlation between these changes and patients' and cycles' characteristics, concluding that it was "HCG administration that leads to endothelial activation regardless of the degree of ovarian response". Through our study, including 85 patients, we believe that it is COH itself, and not HCG, that causes the activation, via the rising E-selectin levels. Regarding the association between ROS in the FF and the IVF outcome, literature evidence is conflicting [31], demonstrating positive [33] and negative [34, 35] links; in addition, the reporting different primary outcomes, the different units of measurement of ROS, and the statistical analysis used, has made the extraction of robust data impossible. Interestingly, Elizur et al. [37] observed a positive correlation between low ROS $[H(2)O(2)]$ levels and high-quality embryos, concluding that the increase in $H(2)O(2)$ level in the follicle is probably associated with ovarian ageing. In our study, no such correlation was

observed, irrespectively of the protocol used (data not shown), a finding that accords with the existing divergent evidence.

Resistin serum levels were found to increase from day 1 of COH until the day of OR, irrespectively of the protocol used. Resistin levels were lower in the FF when the GnRH-agonist protocol was compared with the GnRH-antagonist. This implies that FF resistin seems to be associated with the likelihood of pregnancy, as there was a statistically significant difference in pregnancy rates, favoring the GnRH-agonist protocol. On one hand, a link between low FF resistin levels and GnRH-agonist protocols might have been assumed, but on the other, such correlation did not emerged from the performed analysis, so that this result should be interpreted with caution.

In contrast to our findings for E-selectin, resistin serum baseline levels before the initiation of ovarian stimulation, were associated with the possibility of pregnancy. Notably, ROC analysis revealed that resistin levels greater than 13.5 ng/ml were linked with a 6-fold increase of the odds of a pregnancy, compared to levels below that value; and that was irrespectively of the woman's age, BMI or protocol used. In contrary, Chen et al. [2007] in a case–control study, reported a negative correlation between resistin levels and the number of oocytes retrieved, but similar fertilization rates, in 44 non-PCOS women undergoing IVF; authors suggested a direct link of high levels with poor ovarian response and attributed their findings to the direct effect of inflammatory cytokines on resistin production, cytokines which, per se, were implicated to the etiology of their subfertility [24]. Lu et al. (2005), in a retrospective observational study, found no correlation between serum measurement on day 3 before stimulation and FF levels on fertilization, maturation, miscarriage and clinical pregnancy rates in 20 PCOS and 20 non-PCOS women undergoing IVF; authors concluded that resistin does not have any impact in steroidogenesis and oocyte development in this group of subfertile women [26]. Additionally, Seow et al. [2005] in a prospective clinical trial, found no correlation between serum and FF resistin levels with pregnancy outcome in PCOS women on IVF [25].

We have also observed differences in data published so far, concerning the comparison of resistin levels between serum and FF. Our results oppose to those reported by Seow et al. [25] and Raverchon et al. [28], which indicated lowered resistin levels detected in FF than in plasma in 39 and 9 women [both PCOS and non-PCOS] respectively, suggesting that "resistin does not have a paracrine effect in the process of oocyte development in women undergoing IVF". In our larger sample, we observed higher FF levels (measured in the first follicle aspirated at OR) compared to serum levels during COH, being lower only to those on the day of the OR in the total sample and in the GnRH-agonist group. This leads to an apparent conclusion of the critical role of the resistin in both growth and maturation of the oocyte in subfertile women

undergoing IVF, irrespective of their BMI and cycle characteristics.

The visible limitations of our study are mainly attributed to its nature. The lack of power calculation and equality of the size of the groups studied, blinding and proper randomization are linked with possible unknown confounders and selection bias, while partially explains the difference in pregnancy rates between the two protocols, as the literature is clear [38]. Specifically, the allocation based on the number of patient's file does not represent a formal random component in the sequence generation process, as stated in the Cochrane Handbook for Systematic Reviews of Interventions; the latter would necessitate referring to a random number table or generator [39]. Moreover, residual confounding, such as antral follicle count and response to stimulation, cannot be excluded and this might potentially have an effect on the strength of the association between baseline resistin levels and clinical pregnancy rates.

In terms of clinical limitations, one is that we measured the respective molecules in the first follicle aspirated during OR, using it as a unique sample. Practically, this is done, as the first is one of the bigger ones, and is assumed that it will contain an oocyte. In an ideal study, we should include all aspirates from each follicle individually, but that would necessitate multiple vaginal punctures, something that is totally limited in clinical practice [40]. In our study, follicular selection was carried out using follicular size, assuming that the optimum size for oocyte maturation and fecundation capacity is >18 mm [41].

Conclusion[s]

This prospective study on 85 subfertile women undergoing IVF, using both GnRH-agonist and -antagonist protocols, presents dissimilar results with those reported so far in the literature. E-selectin, resistin and ROS levels are increasing during COH, reaching their highest values at OR, with comparable values measured in the FF at that time. Resistin levels are associated with the likelihood of pregnancy and values >13.5 ng/ml are linked with a 6-fold increase on the odds of a pregnancy. Properly powered and conducted prospective studies are further needed to support these findings.

Acknowledgments The authors would like to thank the staff of the IVF Unit or the 3rd Department of Obstetrics and Gynecology of University of Athens.

Conflict of interest All authors declare no conflict of interest.

Funding There was no finding for the current work. The study is a part of the PhD thesis of the second author.

Authors' roles CS: Study design, interpretation of findings and manuscript drafting.

MA: Study design, data collection and interpretation of findings.

CV: Study design, data analysis and manuscript preparation.

DK: Data acquisition and interpretation of results.

CC: Data acquisition, data collection and interpretation of results.

All authors critically reviewed and approved the final version of the manuscript.

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