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IL-18 and IL-18 binding protein concentration in ovarian follicular fluid of women with “tubal factor” subjected to in vitro fertilization*

Stężenie interleukiny 18 (IL-18) i białka wiążącego interleukinę 18 (IL-18BP) w płynie pęcherzykowym pacjentek z czynnikiem jajowodowym zakwalifikowanych do zapłodnienia pozaustrojowego

Authors' Contribution:

- A Study Design
- B Data Collection
- C Statistical Analysis
- D Data Interpretation
- E Manuscript Preparation
- F Literature Search
- G Funds Collection

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Summary

Introduction:

Composition of follicular fluid is also regarded to be linked to quality of oocytes, fertilization and quality of the embryo. The aim of this study was to investigate the concentration of IL-18 and IL18BP in follicular fluid (FF) in a homogeneous group of women with sterility caused by “tubal factor” subjected to *in vitro* fertilization (IVF) and the relation between concentrate of this cytokine and IVF outcome.

Materials/Methods:

The study group consisted of 83 non-smoking women aged 30.9 ± 3.2 (23.0–43.0) with confirmed (hysterosalpingography and/or laparoscopy) bilateral complete tubal impermeability. Follicular fluid levels of IL-18 and IL18BP were evaluated in 83 patients undergoing *in vitro* fertilization (IVF). Ovarian hormonal stimulation was conducted according to a GnRH antagonist protocol. The measurement of IL-18 and IL18BP in follicular fluid was done using the ELISA method.

Results:

The mean follicular levels of IL-18 and IL18BP were 468.5 ± 357.4 pg/ml and 8611.3 ± 534 pg/ml. The biochemical pregnancy rate was 39.7% (33/83); 22 women became clinically pregnant (26.5%). The implantation rate was 26.7% (36/135). No significant correlation was found between follicular concentrations of IL-18 and age of the patients ($r=0.13$ $p>0.05$), number of metaphase II oocytes collected ($r=-0.11$ $p>0.05$), number of 3-day embryos ($r=-0.157$ $p>0.05$), biochemical pregnancies ($r=0.03$ $p>0.05$), or clinical pregnancies ($r=-0.06$ $p>0.05$). Also there was no significant correlation between IL18BP and age of the patients ($r=0.21$ $p>0.05$), number of metaphase II oocytes collected ($r=0.08$ $p>0.05$), number of 3-day embryos ($r=-0.19$ $p>0.05$), biochemical pregnancies ($r=0.11$ $p>0.05$) and clinical pregnancies ($r=-0.34$ $p>0.05$).

Conclusions:

IL-18 and IL18BP are detectable in follicular fluid but do not determine IVF outcome in women with “tubal factor”. IL-18 and IL18BP are not promising prognostic markers for IVF success in this subgroup of patients.

Keywords:

Interleukin 18 • interleukin 18 binding protein • follicular fluid • in vitro fertilization • cytokines

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Abbreviations **FF** – follicular fluid, **IVF** – *in vitro* fertilization, **ET** – embryo transfer, **IL18** – interleukin 18, **IL18BP** – interleukin 18 binding protein, **MII** – metaphase II oocyte, **EGF** – epidermal growth factor, **hCG** – human chorionic gonadotropin, **ICSI** – intra-cytoplasmic sperm injection, **M-CSF** – macrophage colony-stimulating factor, **MCP-1** – macrophage chemoattractant protein-1, **GM-CSF** – granulocyte-macrophage colony-stimulating factor, **GnRH** – gonadotrophin-releasing hormone, **LIF** – leukemia inhibitory factor, **OPU** – ovarian pick-up, **TNF- α** – tumor necrosis factor alpha, **OHSS** – ovarian hyperstimulation syndrome, **rFSH** – recombinant follicle-stimulating hormone, **rhCG** – recombinant human chorionic gonadotropin, **VEGF** – vascular endothelial growth factor.

INTRODUCTION

The immuno-endocrine network seems to be responsible for both female reproductive success and failure; however, the role of precise cytokine regulation in reproduction is still far from a definite solution. Follicular fluid contains factors responsible for oocyte maturation and ovulation; therefore some disturbances of its composition may affect folliculogenesis [5,11,15,21]. The presence of many pro-inflammatory and immuno-regulatory cytokines, as well as growth factors, was confirmed in follicular fluid including interleukins (IL-1 β , IL-2, IL-6, IL-8, IL-12, IL-18), tumor necrosis factor (TNF)- α , interferons (IFNs), leukemia inhibitory factor (LIF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor (M-CSF), macrophage chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF), suggesting their role in oocyte development, ovulation and regulation of oocyte quality. The results of these investigations have provided the background for research on the influence of cytokines on the reproductive outcome during IVF cycles. The studies were mostly concentrated on the cytokine concentrations in both patients' serum and follicular fluid, and so far have given inconsistent results [2,4,5,6,8,10,11,13,15,17,18,21].

Interleukin 18 (IL-18) is a cytokine that belongs to the IL-1-superfamily. Although initially thought to be produced mainly by macrophages and adipocytes, now it has been found that IL-18 mRNA is expressed in a variety of cells including Kupffer cells, T and B cells, osteoblasts and dendritic cells [19]. IL-18 was believed to be an exclusively pro-inflammatory cytokine able to induce strong Th1 responses in immunocompetent cells, to activate NK cells' and T lymphocytes' killing properties, and to up-regulate the expression of chemokine receptors [24]. Its function was indicated in host defense against severe bacterial, fungal, viral and protozoal infections, as well as in tumor re-

jection. However, recent studies have revealed that IL-18 could also function as a Th2 stimulant. This bidirectional IL-18 activity for naïve T cells depends on the cytokine microenvironment, and in the presence of IL-12 shifts them towards IFN γ -producing Th1 cells, while upon IL-2 stimulation it augments IL-4 and IL-13-secreting Th2 lymphocytes [27,28]. The biological functions of IL-18 are mediated through its receptor (IL-18R). IL-18R resembles IL-1R and shares with it a signal-transduction pathway [1]. Expression of IL-18R was confirmed on both Th1 lymphocytes and NK cells, whereas it was not observed on Th2 lymphocytes [17]. The interaction between IL-18R and its ligand could be modified by the presence of IL-18 binding protein (IL18BP), which blocks the binding of IL-18 to IL-18R. IL18BP mRNA is expressed constitutively in various tissues, and may act as a soluble decoy receptor [20,23].

Expression of both IL-18 and IL-18R was found in the ovaries of pregnant mice [23]. IL-18 has been suggested to favor ovarian folliculogenesis [15,21]. Inactivation of IL-18R with murine monoclonal antibody during ovarian stimulation reduced the number of ovulated oocytes and inhibited the expansion of cumulus cells surrounding the ovum [23]. Significantly higher IL-18 levels were also detected in serum and peritoneal fluid of patients with severe ovarian hyperstimulation syndrome [3]. Furthermore, higher follicular IL-18 concentration was correlated positively with the chance for successful pregnancy, whereas lower levels of IL-18 were found in women with idiopathic infertility [22].

In the opinion of some authors, increased concentration of IL-18 might be a promising prognostic marker for IVF success [16,22].

The aim of this study was to investigate the concentration of IL-18 and IL18BP in follicular fluid in a homogeneous

group of women with sterility caused by “tubal factor” subjected to *in vitro* fertilization (IVF).

MATERIAL AND METHODS

Patient selection and ovarian stimulation

The study group consisted of 83 non-smoking women aged 30.9 ± 3.2 (23.0–43.0) with confirmed (hysterosalpingography and/or laparoscopy) bilateral complete tubal impermeability (so-called “tubal factor”) admitted to the Division of Reproductive Medicine of “Gameta Hospital”, Lodz, Poland, and evaluated prospectively between 2009 and 2010. The Local Ethics Committee on Human Research approved this study, and informed consent was obtained from all patients. Semen samples obtained from the male partners were examined according to World Health Organization criteria, and women having partners with impaired sperm parameters were excluded from the trial. All included couples were administered 1 gram of azithromycin one month before ovarian stimulation. The results of serological and microbiological assays (bacterial vaginosis, *Chlamydia trachomatis*, hepatitis B and C, toxoplasmosis, cytomegalovirus infection, human immunodeficiency virus, lues), as well as routine laboratory tests (leukocytosis, sedimentation rate, serum C-reactive protein) performed just before IVF did not reveal signs of any infection.

Ovarian hormonal stimulation was conducted according to a protocol of gonadotrophin-releasing hormone (GnRH) antagonist (Cetrotide; Merck Serono, Geneva, Switzerland) 0.25 mg daily commenced on stimulation day 6 until the end of stimulation, administered with recombinant follicle-stimulating hormone (rFSH) (Gonal-F; Merck Serono, Geneva, Switzerland) 150 IU given once daily subcutaneously (so-called “short antagonist protocol”). Recombinant human chorionic gonadotropin (rhCG) (Ovitrelle, Merck-Serono, Geneva, Switzerland) 250 µg subcutaneously was administered when at least three follicles having 18 mm diameter each were visualized using trans-vaginal ultrasound. The oocyte pick-up was scheduled 36 h later.

Assessment of oocyte maturity, ICSI and embryo culture

Within 3 hours after oocyte pick-up both the cumulus oophorus and the corona radiata were removed from oocytes using micropipette manipulation after 20 s incubation in a solution of 40 IU/ml hyaluronidase (Hyase, Vitrolife, Göteborg, Sweden) carried out at 37°C. Denuded oocytes were assessed for maturity. Only mature metaphase II oocytes identified by the presence of the first polar body were fertilized. All patients had a standard intra-cytoplasmic sperm injection (ICSI) procedure performed, using commercially available culture media (Vitrolife, Göteborg, Sweden) in a multi-dish under mineral oil. Oocytes and embryos were cultured in an incubator at 37°C in humid atmosphere containing 6% CO₂, 5% O₂ and

89% N₂. Embryo development was evaluated 3 days after fertilization and good-quality embryos were identified by the presence of at least four blastomeres and low fragmentation rate (<20%). The two best quality embryos were transferred for every patient. Biochemical pregnancy was assessed based on the serum hCG on the 10th day after the embryo transfer. Clinical pregnancy was confirmed by the presence of a gestational sac 30 days after blastocyst transfer [29]. The basic characteristics of the study group are presented in Table 1.

Follicular fluid collection and cytokine assay

Follicular fluid (FF) was collected from all matured follicles (>18 mm of mean diameter) during ovarian pick-up (OPU) and then centrifuged at 3000g for 15 minutes to eliminate cells and debris. Supernatant was separated and stored at –20°C until the levels of IL-18 and IL18BP were evaluated. The measurement of IL-18 and IL18BP in follicular fluid was done using commercially available ELISA kits provided by Medical & Biological Laboratories Co (Nagoya, Japan) and R&D Systems (Minneapolis, USA) respectively. All samples were thawed at one time, tested in triplicate and the results were expressed as the mean ± standard deviation (SD). The absorbance was read at 450 nm and 620 nm (reference wavelength). The detection limits were 12.5 pg/mL for IL-18 and 1.6 pg/mL for IL18BP.

STATISTICAL ANALYSIS

Data were analyzed for normality of distribution using the Shapiro–Wilk test. The statistical analysis was performed using unpaired Student’s t-test, Mann-Whitney U test and Spearman test for correlation analysis.

RESULTS

The mean follicular level of IL-18 and IL18BP was 468.5 ± 367.4 pg/ml and 8611.3 ± 533.9 pg/ml respectively. The number of mature metaphase II oocytes was 9.3 ± 6.1 , and the number of good-quality 3-day embryos was 5.2 ± 3.6 (Table 1). The biochemical pregnancy rate was 39.7% (33/83) whereas 22 women (26.5%) became clinically pregnant. On day 3 431 cleavage embryos were obtained and 184 (42%) of them were “top quality” grade A embryos (Table 1). Eighty-three embryo transfers were performed and 135 embryos were transferred. The implantation rate was 26.7% (36/135).

We did not find any significant difference in the number of MII oocytes and 3-day embryos, or the FF concentration of IL-18 and IL-18BP, between the group of 22 woman who achieved and the group of 61 women who failed to achieve biochemical and clinical pregnancy (Tables 2 and 3).

Considering the implantation rate, no significant difference was found in concentration of IL-18 or IL-18BP in the group of patients with positive and negative implantation (Table 4).

Although there was a weak negative correlation between the concentration of IL-18 and IL-18 BP in FF, it was found to be insignificant (Figure 1). There was no significant correlation between IL-18 and IL18BP and positive hCG level ten days after embryo transfer ($r = -0.270$ $p > 0.05$ and $r = -0.124$ $p > 0.05$ respectively). No significant correlation was also found between follicular concentrations of IL-18 and age of the patients ($r = 0.13$ $p > 0.05$), number of metaphase II oocytes collected ($r = -0.11$ $p > 0.05$), number

of 3-day embryos ($r = -0.157$ $p > 0.05$), biochemical pregnancies ($r = 0.03$ $p > 0.05$), or clinical pregnancies ($r = -0.06$ $p > 0.05$). Also there was no significant correlation between IL18BP and age of the patients ($r = 0.21$ $p > 0.05$), number of metaphase II oocytes collected ($r = 0.08$ $p > 0.05$), number of 3-day embryos ($r = -0.19$ $p > 0.05$), biochemical pregnancies ($r = 0.11$ $p > 0.05$), clinical pregnancies ($r = -0.34$ $p > 0.05$) or implantation rate ($r = -0.19$ $p > 0.05$).

Table 1. Follicular fluid IL-18 and IL18-BD concentrations, age, number of MII oocytes, number of 3-day embryos, number of grade A embryos and value of positive hCG in study group

No. of Patients n=83	Mean value	Minimum value	Maximum value	SD
IL18 [pg/mL]	468.5	115.5	2133.7	367.4
IL18BP [pg/mL]	8611.3	7621.7	9907.4	533.9
Age	30.9	23.0	43.0	3.2
MII oocytes	9.3	1.0	31.0	6.1
Number of 3-day embryos (n=431)	5.2	0.0	15.0	3.6
Number of top quality (grade A) embryos in day 3 (n=184)	2.2	1	5	1.1
Positive hcg level 10 days after ET [mIU/ml] (n=33)	88.3	34	180	30.2

Table 2. Follicular fluid IL-18 and IL18-BD concentrations, age, number of M II oocytes, number of 3-day embryos and grade A embryos in the group of women with clinical pregnancy compared to the group of women without clinical pregnancy

Clinical pregnancy	Age	MII	Number of 3-day embryos	Number of top quality (grade A) embryos in day 3	IL-18	IL-18BP
Yes n= 22	30.2 ± 3.2	10.1±5,3	6.1±3.5	2.6±1.3	413.7±381.3	8703.9± 539.2
No n=61	31.2 ± 3.0	9±6.4	4.8±3.5	2.0 ±1	488.3±327.4	8577.8±532.5
P	ns	ns	ns	P<0.05	ns	ns

Table 3. Follicular fluid IL-18 and IL18-BD concentrations, age, number of MII oocytes, number of 3-day embryos and grade A embryos in the group of women with biochemical pregnancy compared to the group of women without biochemical pregnancy

Biochemical Pregnancy	Age	MII	Number of 3-day embryos	Number of top quality (grade A) embryos in day 3	IL-18	IL-18BP
Yes n= 33	30.1 ± 3.1	9.5±6.8	5.2 ±3.3	2.4±1.2	455.7±314	8685.8± 526.3
No n=56	31.1 ± 2.9	9± 4.9	5.1 ±3.2	2.0±1.1	479.3±390	8562.1,8±526.3
P	ns	ns	ns	ns	ns	ns

Table 4. Follicular fluid IL-18 and IL18-BD concentrations, age, number of MII oocytes, number of 3-day embryos and grade A embryos in the group of women with successful implantation compared to the group of women without implantation

No of embryos im- planted	Age	MI	Number of 3-day embryos	Number of top quali- ty (grade A) embryos in day 3	IL-18	IL-18BP
Yes 36/135 (26.7%)	30.2±3	8.6±5	4.6 ±3.1	3.2±1.0	420.7±390	8712± 544
No 99/135 (73.3%)	31.6±3.2	9.5±6.7	5.2 ±3.7	2.4±1.25	490±340	8553 ±544
P	ns	ns	ns	ns	ns	ns

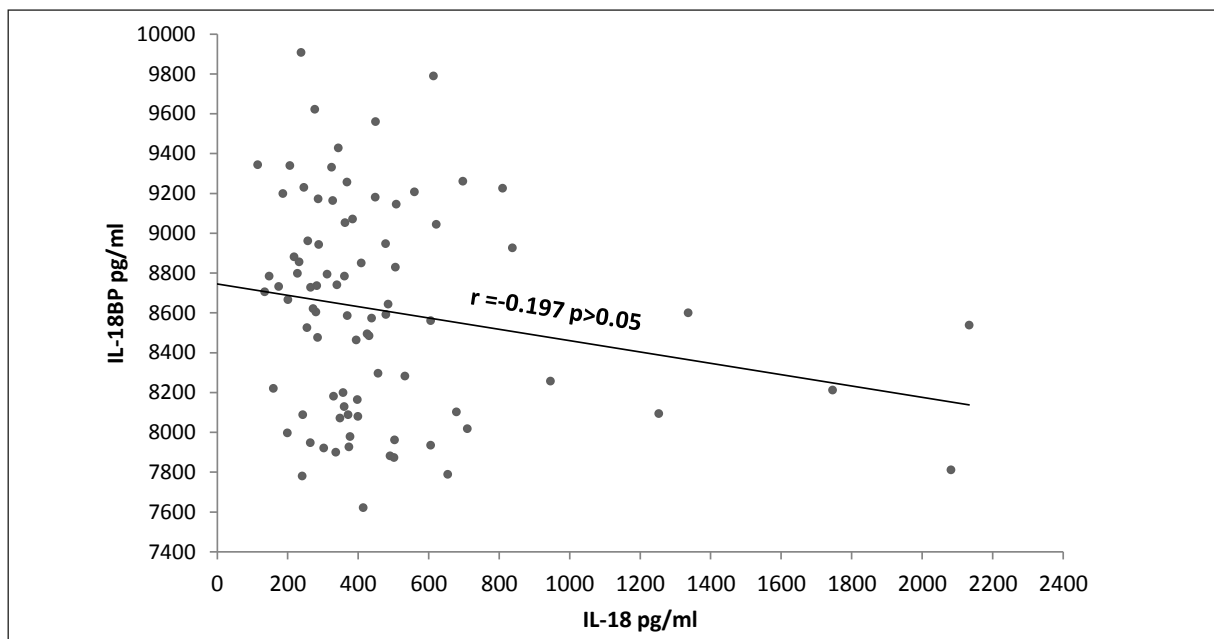


Fig. 1. Correlation between the level of IL-18 and IL18BP in follicular fluid

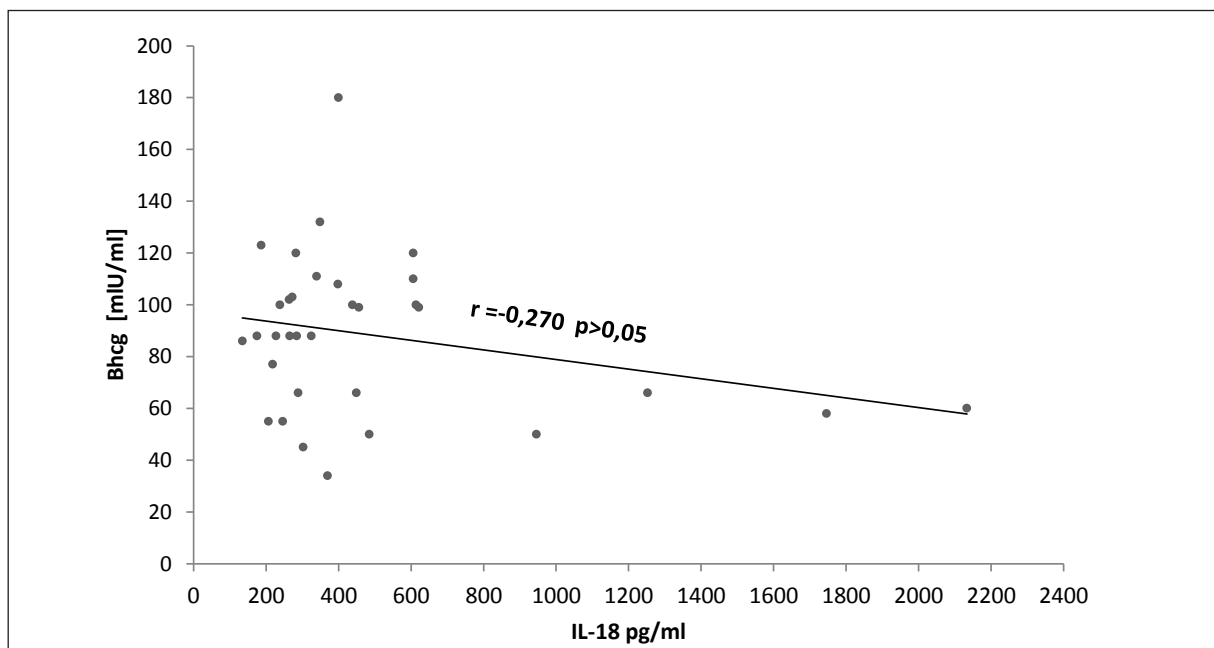


Fig. 2. Correlation between the positive level of hCG in serum and IL-18 in follicular fluid

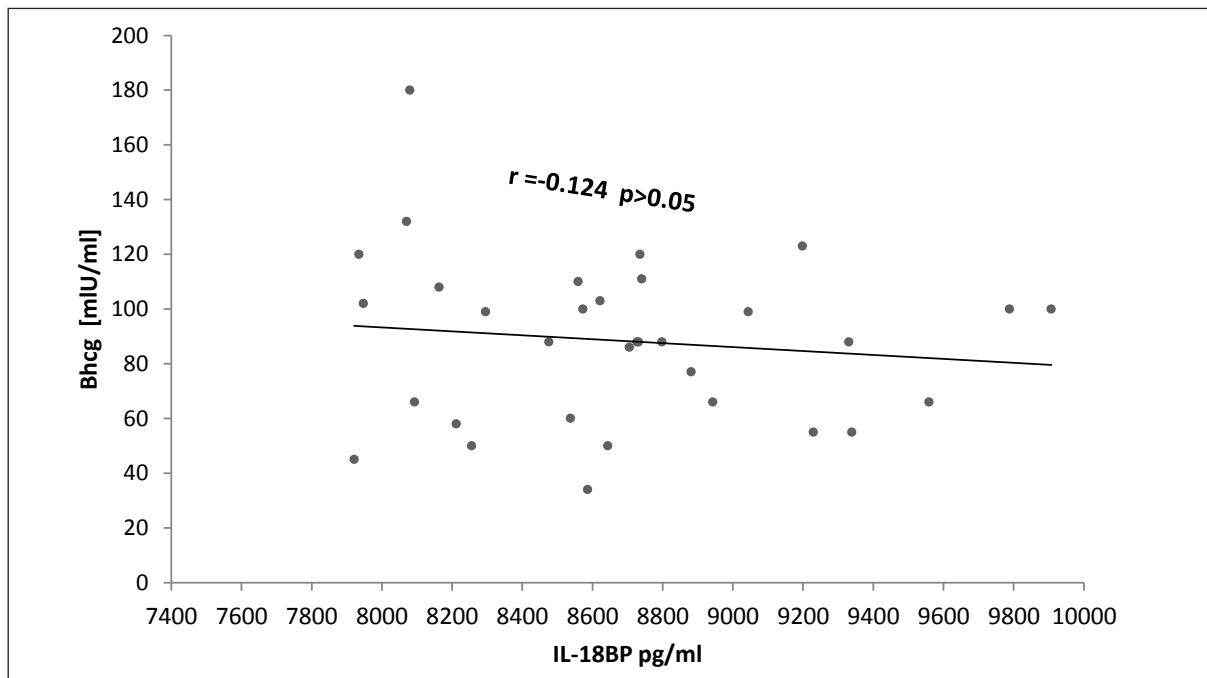


Fig. 3. Correlation between the positive level of hCG in serum and IL18BP in follicular fluid

DISCUSSION

Only a few studies have investigated a possible relationship of IL-18 with IVF outcome. To our knowledge, our study is the first one to investigate both IL-18 and IL18BP in follicular fluid in patients with tubal factor infertility stimulated using a “short antagonist protocol”.

Cytokines inside the ovary seem to be important modifiers of follicular growth and ovulation. The presence of considerable amounts of IL-18 in follicular fluid observed in our study suggests its role in follicular growth and maturation. This suggestion is supported by results obtained by Sarapik et al., who showed that IL-18 was positively correlated with follicle growth [22]. However, IL-18 during reproduction has many faces. Detectable levels of IL-18 were found not only in follicular fluid, but also in uterine luminal secretions and patient’s serum [12,15,25,26]. Increased level of uterine IL-18 was correlated with decreased uterine receptivity and low pregnancy rate. Similarly, IL-18 serum concentration was shown to be higher in women with recurrent miscarriage and was correlated with pregnancy loss [26]. The observed follicular fluid or serum levels of IL-18 also depended on the kind of pathology of the female reproductive tract including endometriosis, polycystic ovarian syndrome (PCOS), and ovarian hyperstimulation syndrome (OHSS) [9].

According to some observations, concentrations of IL-18 could serve as a prognostic factor of both reproductive success and failure. Detectable levels of interleukin-18 in uterine secretions at the time of oocyte retrieval predicted failure of the IVF procedure in the study of Ledee-Bataille et al. [16]. The group of patients positive for IL-

18 had a significantly lower pregnancy rate, multiple pregnancy rate and implantation rate as well. Follicular fluid IL-18 at the time of oocyte retrieval was positively correlated with the number of oocytes and predicted the development of OHSS in high responders [7,9]. A similar conclusion was presented by Barak et al., who found that significantly higher IL-18 levels were detected in serum and peritoneal and pleural fluids of patients with severe OHSS as compared with a control group [3]. The recent study of Sarapik et al. showed a significant positive correlation between follicular IL-18 levels and increased chance for intrauterine pregnancy, number of fetuses detected by ultrasonography and with increased parity [22]. However, our data neither confirmed any correlation between IL-18 concentration and pregnancy outcome in the group of infertile women with “tubal factor” subjected to the IVF procedure, nor showed any correlation of IL-18 with the number of mature MII oocytes and good-quality embryos. Lack of a correlation of intrafollicular IL-18, IL-18BP, and the IL-18/IL-18BP ratios with the number of oocytes retrieved and transferred, embryo grades or pregnancy ratios was observed in obese, overweight, and non-obese patients by Kilic et al. [12], supporting our observations. The Sarapik et al. study was performed on a heterogenic group of infertile patients (tubal factor infertility, PCOS, endometriosis, unexplained infertility), and the authors collected the greatest group of patients from couples with exclusively male infertility [22]. In contrast, our group of patients was homogeneous according to the reason for infertility, which was the strength of our study. Moreover, the methodology of evaluation of follicular fluid samples differed in both studies. It was shown that the assay used for quantification of cytokine in follicular fluid

might influence the final result [14]. Use of an ELISA assay might be considered in this context as a weakness of our study. Moreover, the results of different studies could be hardly comparable due to the different stimulation protocols used. In spontaneous cycles, values of IL-18 in follicular fluid are lower (38.2 ± 25.1 – 50.2 pg/ml) than during stimulation [25]. In the study of Gutman et al. [9] all women were stimulated using GnRH agonist in a so-called “long protocol”. The mean level of IL-18 was lower in comparison to our study (228 ± 208 pg/ml vs. 468.5 ± 357.4 pg/ml). The probability that type of ovarian stimulation influences and modifies cytokine synthesis in follicular fluid including IL-18 cannot be rationally excluded.

IL-18 binding protein is regarded as a natural inhibitor of IL-18 activity. We found that a negative correlation did exist between IL-18 and IL18BP concentrations in follicular fluid, but it was insignificant. Therefore, it is probably not IL18BP that is mainly responsible for regulation of IL-18 inside the growing follicle. The role of different cytokines in this process still needs further elucidation.

In conclusion, IL-18 and IL18BP are detectable in follicular fluid but do not determine IVF outcome in women with “tubal factor”. IL-18 and IL18BP do not seem to be promising prognostic markers for IVF success in this subgroup of patients.

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