# Determination of the main factors associated with the outcome and the comlpications of in vitro fertilization

**Doctoral (Ph.D.) thesis**

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## *1. Introduction*

World-wide, the number of people taking part in fertility treatments and in assisted reproductive techniques is growing. Currently approximately 3-4% of all births are such births and this number continues to increase. With the widespread use of assisted reproductive techniques, especially in vitro fertilization, there are more and more pregnancies conceived by these methods. However, we have to consider the frequent incidence of potential side effects of IVF treatment. There are considerable measures being taken to increase the success rate and in order to minimize the side effects.

For this reason there have been several studies in the past few years dedicated to the study of intraovarian regulation. Several proteins with roles in pathogenisis have been examined, however newer data emphasizes substances such as activin, inhibin, insulin/like growth factor (IGF) I and II, epidermal growth factor, IGF-binding proteins, intraovarian renin-angiotensin system, oxytocin, and opiates. Recently, the data has been supplemented with cytokins and interleukins, as well as with the roles of several (neuron)paracrine factors.

Unfortunately, we know very little about the roles these substances play in the development of diseases related to the regulation of ovarian function, such as, polycystic ovarian syndrome, or severe, occasionally lethal iatrogenic condition, ovarian hyperstimulation syndrome (OHSS).

OHSS is a systemic disease triggered by vasoactive products released from hyperstimulated ovaries. According to previous studies, a mild form of OHSS is common, affecting up to 33% of IVF cycles. Approximately 3-8% of IVF cycles are complicated by moderate to severe OHSS. The majority of severe OHSS cases are experienced following IVF treatment, but the syndrome can occur after any form of supraphysiological ovarian stimulation, including clomiphene and gonadotropin ovulation induction.

The incidence of OHSS has increased in young women, in patients with polycystic ovaries, and in cycles, where conception occurs, particularly in cases of multiple pregnancies. Leading symptoms and signs of this severe condition include: free fluid collection in serous cavities, skin-edema, hemoconcentration, stimulation of the renin-angiotensin and sympathetic nervous systems, and that of antidiuretic hormone production. Increased heart-rate and cardiac output are also observed. It is characterized by increased capillary permeability, leading to leakage of fluid from the vascular compartment, causing free fluid accumulation and intravascular dehydration.

While the exact mechanism of hyperstimulation is still unknown, it is certain, that it is accompanied by peripheral artery vasodilation, increased vascular permeability, and thrombocyte activation. Factors belonging to the renin-angiotensin system, cytokines, including the interleukins (IL-8 and IL-6), tumor necrosis factor alpha, endothelin-1, and vascular endothelial growth factor (VEGF) are thought to trigger increased vascular permeability. A growing body of evidence suggests that increased platelet activation strongly correlates with VEGF levels. Moreover, activated platelets in OHSS can release histamine, serotonin, platelet-derived growth factor, or lysophosphatidic acid (LPA), which might further potentiate the patho-physiological cascade leading to OHSS. Among them, LPA, a biologically active phospholipid has recently been found to mediate excessive IL-8 and IL-6 secretions from multiple corpora lutea of superovulated ovaries.

Diverse clinical manifestations of the syndrome include: a tendency to develop phlebo-thrombosis, renal and liver dysfunction, and adult respiratory distress syndrome (ARDS), all responsible for serious morbidity associated with OHSS.

Currently we don't know the exact roles of factors found in follicular fluid (acetilcholin, serotonin, histamin) in intraovarian regulation and their relationship to LPA receptors, but regarding our groups previous work we are well aware of the fact that they greatly modify the function of the granulose cell. At the same time it is known that thrombocytes have an important role in ovarian inflammatory processes. It has also been shown that pituitary adenylate cyclase-activating polypeptides (PACAP) play a role in inflammatory processes by decreasing the levels of tumor necrosis factor alpha (TNF alpha), which is in close relation to the endocrine system. This leads us to assume that the previous function also has an impact on reproduction biology.

Previous studies have shown that VEGF mRNA expression is in connected to the vasoproliferation of the ovaries, and several data leads us to believe that the endogenous inhibitor of nitrogen oxide synthase, asymmetrical dimetilarginin (ADMA) modifies the VEGF levels of endothelial cells.

The methods for examining intraovarian regulatory mechanisms are limited. It is possible to use in vitro superfusion systems, such as measuring the levels of effected peptides in granulosa cells, or from human secretion, for example during in vitro fertilization we can measure the concentration of these factors from the "by-product", follicular fluid.

Another option is to measure the effect of a know compound (for example aspirin) during randomized tests, where the results can lead to indirect conclusions based on the processes taking place in the ovaries.

The diagnosis is usually straightforward: it is based on the previous history of ovarian stimulation followed by the typical symptoms of abdominal dystension, abdominal pain, nausea, and vomiting. This is then further confirmed by laboratory parameters and ultrasound imaging.

To determine the prognosis, depending on the time of onset, classification of OHSS into 'early' and 'late' types may be useful. OHSS presenting within 9 days after the ovulatory dose of human chorionic gonadotropin (hCG) is likely to reflect an excessive ovarian response along with the precipitating effect of exogenous hCG administered for final follicular maturation. OHSS presenting after this period reflects the endogenous hCG stimulation from an early pregnancy. Late OHSS is more likely to be severe and lasts longer than early OHSS. Although utilizing dopamine as therapy is usually effective the main goal remains to be prevention of OHSS.

On the basis of this theory aspirin (acetyl-salicylic acid) administration may be an effective prophylaxis for patients at high-risk for ovarian hyperstimulation. Earlier studies have shown a beneficial effect of low-dose aspirin therapy during IVF (9). In IVF centers the main goal of aspirin therapy was to improve pregnancy rates. Based on the theory, that superovulation treatment may induce platelet hyperstimulation, which is associated with OHSS, and that aspirin therapy may inhibit this effect, we started the administration of aspirin as a preventive measure. In this paper we describe our results with the administration of aspirin in controlled ovarian hyperstimulation cycles at our IVF-center with regard to the development of OHSS.

In our tests ovarian hyperstimulation syndrome and the role of VEGF was examined as well as the effect of aspirin during in vitro fertilization treatment. We studied the presence of pituitary adenylate cyclase-activating polypeptides (PACAP) and its concentration in follicular fluid and the role of different arginine derivatives during in vitro fertilization treatments.

## *2. Aims*

### *2.1. Possibilities for testing ovarian hyperstimulation syndrome prophylaxis*

Based on our theory that ovulation is an inflammatory process associated with thrombocyte activation, the administration of aspirin (acetylsalicylic acid) could be a useful prophylaxis in light of OHSS. Earlier studies have shown the beneficial effect of low-dose aspirin therapy during IVF treatment. In IVF centers the main goal of aspirin therapy was to improve pregnancy rates. Based on this theory, that superovulation treatment may induce platelet hyperstimulation, which is associated with OHSS, and that aspirin therapy may inhibit this effect, we started the administration of aspirin as a preventive measure. Thus at our Clinic's IVF Center patients receiving superovulation treatment were administered aspirin as a prophylaxis during their treatment. Our goal was to measure the success rate of preventing the development of OHSS by inhibiting the function of thrombocytes with retrospective and prospective testing.

### *2.2. Detecting PACAP 38 in follicular fluid*

Although it has been two decades since its discovery revealed that PACAP is much more than just a hypothalamo/hypophyseal peptide, its role in the endocrine system is still the focus of research.

Ovarian follicular fluid is the product of granulosa and theca cells as well as plasma filtrated through the wall of the developing follicle. It functions as a culture medium for the developing oocyte. Since PACAP has been shown to play a role in follicular development, it was of interest to examine whether the peptide can be found in the follicular fluid and if present, if it is possible to measure the concentration and the ovarian function, in other words, the response to the superovulation treatment, and if there is any connection to follicular development.

### *2.3. Detecting arginine derivatives as a measure of IVF treatment success*

Our goal was to determine the derivatives of l-arginine and methylarginine (ADMA, SDMA és MMA) in the follicular fluid of women receiving in vitro fertilization. Furthermore our

goal was the find clinical correlations between these biochemical markers and the result of the IVF treatment. We also examined the ratio of l-arginine to ADMA, as a feature of NO production and bioactivity, as well as the recently introduced arginine-methylation index (Arg-MI) in order to win relevant clinical information with regards to the interaction between follicular fluid and the oocyte/embryo.

## *3. Materials and methods*

### *3.1. Testing ovarian hyperstimulation syndrome prophylaxis*

Patient enrollment into IVF treatment was approved by two independent physicians. Between January 1, 2000 and December 31, 2006 we started 3154 IVF cycles, of which in 2425 cases we administered gonadotropin-releasing hormone (GnRh) agonist, and in 729 cases GnRh antagonist. We didn't include patients receiving GnRh antagonists in our study because they are less likely to develop OHSS because in their case we didn't administer hCG before ovulation.

In 62% (n=1503) of the 2425 GnRh agonist cycles we administered low-dose (100mg/day) aspirin therapy, in 38% of the cycles (n=922) no aspirin treatment was given. Aspirin was also randomly given to patients and was started on the 1st day of the menstrual cycle, when IVF was performed.

GnRh agonist triptorelin was used in either long or short protocol, and the stimulation was performed with individual dosages of rFSH, varying from 100 to 225 IU per day depending on the follicular maturation. The starting dose was determined according to their BMI and age. For patients with a previously known weak response the dosage was increased to a maximum of 300–350 IU daily. The follicular maturation was determined by ultrasound examination from the 6th day of the cycle, every other day. We changed the amount of the administered gonadotropins individually according to the size of the follicles. Ovulation was induced by injection of 250μg of hCG when at least two follicles exceeded 17mm in diameter, and aspiration of follicular fluid was performed 36 hours later by ultrasonography-guided transvaginal puncture under routine intravenous sedation.

Aspirin administration was continued until menstruation or a negative pregnancy test, or until ultrasonographic detection of embryonic cardiac activity. Patients were divided into two groups according to their chances of developing OHSS (Group 1, high-risk, and Group 2, low-risk patients). Criteria for risk assessment included the presence of prior history of OHSS, polycycstic ovaries and an age less than 30.

Aspirin was administered at a dose of 100mg per day in 1503 cases of the 2425 induced cycles.

### *3.2. Detecting PACAP 38 in follicular fluid*

We used mass spectrometry to detect PACAP38 in the follicular fluid and we used radioimmunoassay (RIA) to measure its levels. Follicular fluid was collected from female volunteers (aged between 20 and 35, $n$=40) by follicular puncture after controlled ovarian hyperstimulation during the in vitro fertilization procedure.

The peptidase inhibitor aprotinin was added to all samples (30 µl/ml), and a 100µl of the follicular fluid sample was centrifuged at 10000 rpm for 5 minutes, followed by the addition of 10µl of 72% trichloroacetic acid and 100µl of $H_2O_2$ to 90µl of the supernatant. The samples were centrifuged at 13000rpm for 10 minutes after precipitation.

Identification of PACAP38 was performed with MALDI TOF/TOF MS. In a few words, the mass spectrometer used in this work was an Autoflex II TOF/TOF (Bruker Daltonics) operated in the linear detector for MALDI TOF or LIFT mode for high-energy collision-induced decay MALDI TOF/TOF with an automated mode using the FlexControl software. The ions were accelerated under delayed extraction conditions (200 ns) in positive ion mode with an acceleration voltage of 20.00kV. The instrument uses a 337nm pulsed nitrogen laser, model MNL-205MC (LTB Lasertechnik Berlin GmbH). External calibration was performed in each case using Bruker Peptide Calibration Standard (#206 195 Peptide Calibration Standard, Bruker Daltonics). Protein masses were acquired with a range of $m/z$ 1000 to 10000. Each spectrum was proceeded by accumulating data from 200 consecutive laser shots for standard PACAP38 solution and 1000 for follicular fluid. The Bruker FlexControl 2.4 software was used to operate the instrument and the Bruker Flexanalysis 2.4 software for spectrum evaluation

Follicular fluid was collected as described above in order to test the levels of PACAP. The samples were weighed and centrifuged (12000rpm, 4°C, 30 minutes), and the supernatant was further processed for RIA analysis of PACAP38-like immunoreactivity, as previously described.[16] Briefly, the conditions were the following: antiserum: PACAP38: '88 111-3' (working dilution 1 : 10000), tracer: mono-125I-labeled ovine PACAP24-38 prepared in our laboratory (5000cpm/tube), standard: ovine PACAP38 was used as a RIA standard ranging from 0 to 1000fmol/ml, buffer: the assay was prepared in 1ml of 0.05mol/l (pH 7.4) phosphate buffer containing 0.1mol/l sodium chloride, 0.25% (w/v) BSA and 0.05% (w/v) sodium azide. Incubation time: 48–72 hours incubation at 4◦C. Separation solution: charcoal/dextran/milk powder (10 : 1 : 0.5g in 100 ml distilled water).

### *3.3. Detecting arginine derivatives as a measure of IVF treatment success*

Our study was performed between October 1, 2008 and December 31, 2008. During this time period we started 125 unselected IVF cycles, in 108 cases of them we performed transvaginal ultrasound guided aspiration of follicular fluid. In the remaining 17 cycles the stimulation was unsuccessful. We examined the length of the period in the IVF program, the age, the BMI of the patients and the indication of the IVF. The patients were on an unrestricted normal diet with folic acid supplementation of 0.8mg per day, from the first day of the menstrual cycle and they were all non-smokers.

### *3.3.1. Collection of follicular fluid*

Oocyte collection was performed using Sonoace 6000C two dimensional real time ultrasound scanner equipped with 4-8 MHz endovaginal transducer. The transducer was covered with sterile gel and barrier and original puncture guide was applied on it. The vagina was disinfected with Octanisept (Schülke & Mayr GmbH) and the stimulated ovaries were visualized by the transducer kept in the posterior vaginal wall. A 35cm long, 1.4mm in diameter aspiration needle was inserted through the puncture guide along with the transducer. The follicles were punctured and follicular fluid was retrieved. Only a single puncture was administered and the tip of the needle was drawn back to drain each follicle. The oocyte collection was performed in G-MOPS™ medium (Vitrolife®). Follicular fluid from individual follicles was aspirated and after collecting the oocytes the fluid was centrifuged for 10 minutes at 1500 r.p.m. and the supernatants were frozen and stored at –70°C for future analysis. Fractions of FF with macroscopic blood contamination were excluded.

### *3.3.2. Fertilization methods*

The oocytes were selected according to the fertilization methods. We performed the fertilization with Intracytoplasmatic Sperm Injection (ICSI) depending on the andrological status (sperm count less then 20M/ml), the maternal age (>35) and the number of the previous IVF cycles the patient had before (>2). The oocytes selected for ICSI were denuded with hyaluronidase and were assessed for maturity. Only metaphase II oocytes, identified by the presence of the first polar body, were chosen for fertilization. ICSI was performed 3–6 hours after oocyte recovery in the medium G-MOPS ™ (Vitrolife®). The remaining oocytes were fertilized with the conventional IVF method in a bicarbonate buffered medium (G-IVF ™, Vitrolife®). Fertilization was assessed 24 hours after in the medium G-1™v5 (Vitrolife®).
Embryo transfers were done 3-5 days after the oocyte retrieval. From day 3 to blastcyst stage we use the medium G-2™v5 (Vitrolife®). None of the culture mediums contained folic acid. The G-IVF ™ doesn't contain methionine while the other mediums (G-MOPS™ , G-1™v5 , G-2™v5 ) do.

According to the patients requests we transferred one, two or three embryos. Cryopreservation of the remaining embryos was performed at this stage according to the Hungarian law.

Progesterone supplementation was provided with 300 mg of progesterone 3 times a day (Utrogestan; Lab.Besins International S.A.®).

To evaluate the success of the treatment a transvaginal ultrasound examination was performed 21 days after the embryo transfer to detect the gestational sac.

*3.3.3. Laboratory measurements*

L-arginine, ADMA, SDMA and MMA concentrations in the follicular fluid were determined with liquid chromatography-tandem mass spectrometry (LC-MS-MS) as previously described. The intra-day precision was 4.5% for l-arginine, 5.5% for ADMA, 3.9% for SDMA and 4.0% for MMA. The respective values for inter-day precision were 4.7%, 7.7%, 4.9% and 9.6%. The routine biochemical parameters were measured by using standard laboratory procedures. The integrated index of arginine methylation was calculated according to the formula: Arg-MI = (ADMA + SDMA) / MMA as previously mentioned.

*3.3.4. Statistical analyses*

All statistical analyses were performed with SPSS, version 17.0 (SPSS Inc. Chicago, Ill. USA).

We performed a $\chi^2$ probe during the processing of the data gathered in the OHSS prophylaxis clinical study. In the first group we employed the Yates correction while comparing the treated and non-treated subgroups as a supplementation to the $\chi^2$ probe.

Using the data gathered while testing for the presence of PACAP38 in follicular fluid we examined the average of PACAP concentration and the standard deviation (SD) of the derived oocytes and follicular fluid. We divided the concentration of PACAP and number of oocytes with the median number, and with this we were able create 3 groups: high concentration of PACAP (hP), high number of oocytes (hO) and low concentration of PACAP/low number of oocytes (lP-lO). The data from these groups were analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks test.

We evaluated the normality of the data from examining arginine derivatives using the Kolmogorov-Smirnov test. Correlations between variables were assessed by applying the non-parametric Spearman's rank correlation. ANOVA analysis of variance was also used when appropriate. Variables are presented as mean ± SD. A difference of $p<0.05$ was considered significant.

## *4. Results*

### *4.1. Options for ovarian hyperstimulation syndrome prophylaxis*

Among those, who received aspirin, 52% (n=780) were in the high-risk group, and 48% (n=723) in the low-risk group, respectively. Among the 922 receiving no aspirin, 45% (n=412) and 55% (n=510) of patients were in the high-risk and low-risk groups, respectively.

Out of 2425 cycles severe or critical OHSS was observed in 1.8% (n=45). These patients were admitted to the hospital for intensive care and the treatment of dehydration and hemo-concentration, and to proceed with the transvaginal aspiration of free fluid collection through the Douglas pouch as well. In severe OHSS cases aspirin therapy was continued, and supplementary low-molecular weight heparin (LMWH) therapy was implemented for thromboprohylaxis.

Among those treated with aspirin (n=780) only 2 patients presented with severe or critical OHSS in the high-risk group (0.25%). Among patients receiving no aspirin treatment and categorized in the high-risk group (n=412), OHSS was detected in 43 cases (8.4%, $p<0.001$).

In the group with low-risk patients receiving aspirin prophylaxis (n=723) no OHSS was recorded during the IVF cycles. No OHSS was detected in the low-risk, untreated group (n=510) either.

### *4.2. The presence of PACAP in follicular fluid*

#### *4.2.1. Proving the presence of PACAP*

During our study we examined human follicular fluid with MALDI TOF mass spectrometer along with the PACAP standard. We detected PACAP-38 quasi-molecular ion (MW: 4534.6 Da) in both the standard and in the 40 follicular fluids. Following this we performed the PACAP38 peak fragmentation using MALDI TOF/TOF. The results of the test for the y fragments match the available PACAP38 parent ion z fragments and amino acid sequence of previous studies.

#### *4.2.2. Number of follicles following superovulation treatment.*

During our study the average oocyte number was 8.08 (±6.02), numbers ranging from 0 to 30 with a median value of 6.5.

#### *4.2.3. Determining the concentration of PACAP in follicular fluid.*

The average concentration of PACAP during our study was 143.58 (±110.78), with a value between 28.0 and 690.0fmol/ml and with a median value of 107.75fmol/ml.

#### *4.2.4. Examining the correlation between PACAP concentration in follicular fluid and the number of obtained oocytes*

We created groups using a cut-off value of 290fmol/ml for PACAP concentration and 14 oocytes/patient for the obtained number of oocytes: high concentration of PACAP (hP); high number of oocytes; low PACAP concentration-low number of oocytes (lP-lO).

The median value of PACAP concentration in groups hP (n=12), for hO (n=17), and lP-lO (n=103) was 411.2 fmol/ml (312.5-492.0 fmol/ml), 106.5 fmol/ml (61.0-180.5 fmol/ml) and 101.0 fmol/ml (72.7-139.0 fmol/ml) respectively. The differences between the median values among these groups are statistically significant.

Using the above mentioned cut-off values and groups for the number of obtained oocytes, the median values for these groups are hP (n=12), hO (n=17) and lP-lO (n=103) and 5.5 (4.0-10.0), 19.0 (15.0-21.7) and 5.0 (3.0-10.0 respectively. The differences between the median values among these groups are statistically significant.

*4.2.5. The Evaluation of OHSS*

During our study we found low cases of OHSS in 3 cases. In these 3 cases the concentration of PACAP in the follicular fluid and the number of obtained oocytes were the following: 1.: 109fmol/ml, 6/patient; 2.: 312fmol/ml, 8/patient; 3.: 79fmol/ml, 20/patient. The median values of these 3 patients in the + first and third quartile was 109.0 fmol/ml (86.5-261.25fmol/ml) for PACAP and 8.0 (6.5-17.0) regarding the number of oocytes.

***4.3. The correlation between arginine derivates and the result of IVF treatment***

The patients participated in our IVF program for a period of 3-34 months (mean ± SD 14.1 ± 5.6 months), they're ages ranged from 22-41 years (mean: 33.6 ± 5.5 years) and had a BMI of 18.1-38.3 (mean: 24.0 ± 4.6).

They presented with the following main infertility diagnosis: andrology reasons 34 (31.5%), damaged or blocked Fallopian tubes 24 (21.4%), severe endometriosis 20 (18.5%) and unexplained infertility 30 (28.6%). These latter patients experienced six unsuccessful intrauterine inseminations previously.

Using visual binning, data was grouped into two distinct categories according to the total number of oocytes obtained by FF aspiration. FF with oocytes equal to or less than 9 was compared with FF containing oocytes 10 or more.

ANOVA analysis of variance revealed that the higher number of FF oocytes was associated with significantly more oocytes fertilized either conventionally or by ICSI, with more mature oocytes for ICSI and with more embryos conceived either by IVF or ICSI.

When components of the l-arginine/NO system were binned in a similar way (total oocyte number: ≤9 versus >9) the reduced number of FF oocytes were found to be associated with

elevated FF l-arginine, ADMA and MMA contents. In case of SDMA this association just fell short of statistical significance, whereas the l-arginine/ADMA ratio and arg-MI appeared to be independent of the number of FF oocytes.

Binning the results according to the number of embryos (≤6 versus >6) provided further convincing evidence that l-arginine and its methylated products had an adverse influence on the success rate of fertilization. Specifically, a lower embryo number was associated with significantly higher levels of FF l-arginine, ADMA, SDMA, MMA and Arg-MI, but not with l-arginine/ADMA ratio, the estimate of NO production/bioavailability.

These observations are strongly supported by Spearman's rank correlation analysis which yielded significant inverse relationships of IVF embryo number to FF l-arginine ($r=-0.507$, $p<0.001$), ADMA ($r=-0.356$, $p<0.024$), SDMA ($r=-0.347$, $p<0.028$), MMA ($r=-0.449$, $p<0.004$) and to l-arginine/ADMA ratio ($r=-0.328$, $p<0.031$). By contrast, Arg-MI was directly related to the number of IVF embryos ($r=0.426$, $p<0.006$). In addition to the number of IVF embryos, the IVF oocytes were also related inversely to ADMA ($r=-0.202$, $p<0.037$) and MMA ($r=-0.384$, $p<0.012$) and positively to arg-MI ($r=0.450$, $p<0.003$).

Not surprisingly, there was a strong positive correlation between FF l-arginine and its methylated products. Namely, increased rate of free l-arginine production appeared to be associated with enhanced release of ADMA ($r=0.377$, $p<0.000$), SDMA ($r=0.526$, $p<0.000$) and MMA ($r=0.446$, $p<0.000$). Moreover, the calculated l-arginine/ADMA ratio was positively ($r=0.803$, $p<0.000$), the Arg-MI, however, was negatively related to l-arginine ($r=0.246$, $p<0.011$).

## *5. Discussion*

### *5.1. Aspirin as a prophylaxis for ovarian hyperstimulation syndrome*

OHSS is a severe, potentially lethal iatrogenic condition triggered by vaso-active products released by hyperstimulated ovaries. The number of patients presenting with OHSS is gradually increasing as the number of IVF cycles performed is greater.

OHSS is characterized by increased capillary permeability triggered by VEGF, which correlates with increased platelet activation. Activated platelets release histamine, serotonin, platelet-derived growth factor, or LPA. These substances might further drive the patho-physiological cascade leading to OHSS. The exact mechanism is still unknown but several factors play a role in the development of OHSS by directly or indirectly affecting VEGF. hCG increases the expression of VEGF in human granulose cells as well as the concentration levels of VEGF serum. The rapidly rising hCG is the reason a pregnancy might run a more severe course. The activation of thrombocytes during the induction of ovulation also play a role in the pathomechanism of OHSS. Based on these, we hypothesized that aspirin therapy might prevent this syndrome. To prove this, we randomly administered aspirin to high-risk and low-risk patients undergoing IVF-protocol and determined the occurrence of OHSS in each group.

In our study, among those patients treated with aspirin there were only 2 cases of severe or critical OHSS (0.25%). Both patients were classified in the high-risk group. However, among those high-risk patients, those who did not receive prophylactic aspirin treatment 43 patients (8.4%) presented with OHSS. No OHSS was encountered in the low-risk groups. Since there was a significant difference in the occurrence of severe OHSS between the aspirin-treated and non-treated high-risk groups, this led us to the conclusion that aspirin had an overall beneficial effect in the prevention of OHSS. Furthermore, since mild OHSS symptoms could be observed in the high-risk, aspirin-treated group one might conclude, that administration of aspirin may not only prevent the development of severe or critical OHSS but, it might significantly decrease the risk of development of potentially serious clinical manifestations of the disease.

Analyzing pregnancy outcomes of the study groups we could not observe any significant difference. Results of other authors are similar. However, in accordance with another study by Moini et al. we observed a difference between the incidence of severe OHSS in the aspirin-treated and non-treated groups. Nevertheless, only two of our OHSS patients had previously received aspirin. Based on our findings we strongly recommend the use of low-dose aspirin therapy to prevent OHSS or to reduce the severity of symptoms in all cases associated with high-risk of that severe complication of ovulation induction.

### *5.2. The presence of PACAP38 in follicular fluid*

During our study we found PACAP38 in all of our examined human follicular fluid. The follicular fluid served as a medium for the growing oocyte and played an important role in the morphological and functional development of germinal cells. We can detect PACAP in all granulosa cells of large mature follicles regardless of their developmental stage. Smaller levels are also expressed in immature antral and preantral follicles. They have also shown PACAP receptors to be present in maturing follicles. PACAP and PAC1 receptors have been found in the corpus luteum. It is likely that the peptide plays a role in the proliferation of primordial germ cells, in the cyclic excretion and in the initiation of the development of immature follicles, in the meiotic development of oocytes, and in the production of occyte hormones and enzymes. During our study we detected PACAP in follicle fluid. This confirms our hypothesis that PACAP plays an important role in follicular fluid as a culture fluid for developing oocytes. However further study is necessary to understand the finer physiological mechanisms.

The results of our study to determine the concentration of PACAP, not only corroborated with previous results that PACAP can indeed be found in human follicular fluid, but also gave light – to our knowledge for the first time – of the relation between PACAP concentration and the response of the oocyte to gonadotropins.

During our study we established two cut-off values, one for the PACAP concentration in follicular fluid, the other for the number of maturing oocytes. Each time the number of obtained oocytes from a patient exceeded 14, the PACAP concentration was below 290 fmol/ml, with a median value of 106.5 fmol/ml. In addition in each case when the PACAP concentration exceeded 290 fmol/ml (median value of 411.2 fmol/ml) the number of obtained oocytes/patient was under 14. The difference in the concentration of PACAP in both groups, as well as in the number of obtained oocytes, is statistically significant.

These values point our attention in the direction of the pathomechanism of Ovarian Hyperstimulation Syndrome. It had already been established earlier that OHSS more likely forms in patients receiving superovulation treatment, who have significantly more follicles on days when hCG was administered.

According to a previous significant study the critical number of follicles during the administration of hCG when considering the development of OHSS was 13. This cut-off value coincides with our findings, where significantly lower levels of PACAP were found in the follicular fluid. This could also mean that higher levels of PACAP concentration in follicular fluid could be an indicator when it comes to the maturation of follicles, while low concentrations of PACAP indicates a risk of developing OHSS.

A direct link between the number of follicles and the concentration of PACAP is unknown, the data regarding OHSS are limited (for example: in case of slight symptoms they do not go to the doctor), so we can't jump to conclusions but it is defiantly worth paying attention to the fact that two out of three patients with OHSS have low levels of PACAP while one out of three have a concentration level that falls in the first quarter (312 fmol/ml).

To the best of our knowledge and the literature available, this is the first study that shows a connection between the levels of PACAP in follicular fluid and the number of obtained oocytes.

The exact role of PACAP in this process is still unknown, however as previously discovered, the effect of the peptide on hormone production leads us to hypothesis its role in oocyte maturation, follicle development. In order to learn more details about this connection further studies are necessary.

### *5.3. The connection between the NO system and the outcome of IVF treatment.*

The present study demonstrated that in women undergoing IVF treatment their follicular fluid contained the major elements of l-arginine/NO system including l-arginine, ADMA, SDMA and MMA. Furthermore, increased activity of the l-arginine/NO system proved to have an adverse affect on the reproductive processes as reflected by the elevated levels of l-arginine and methylarginines in the follicular fluid in association with a reduction in the number of oocytes, number of embryos conceived either traditionally or by ICSI, and in IVF outcome.

Studies attempting to define the role of follicular fluid NO in oocyte maturation, fertilization and in embryo development have shown the expression of all NOS isoforms in various ovarian cells during follicular development and in preimplantation embryo. In this regard it is

to be noted, that while single-NOS knockout mice encountered no reproductive abnormalities, double knockout mice (iNOS/eNOS, eNOS/nNOS, iNOS/nNOS) were preferentially lost during early embryonic development. Additionally, when NOS inhibitor L-NA and/or L-NAME were added to the culture media developmental arrest of the embryo could be induced. These inhibitory effects, however, could be reversed by the addition of NO donor, or the second messenger cGMP analogues.

In our present study FF NO or its stable metabolites –nitrite and nitrate- were not measured. Instead, the l-arginine/ADMA ratio as an estimate of NO production/bioavailability was calculated. Although it has limitations, it appeared to be unrelated to the number and maturity of oocytes, number of embryos conceived conventionally or by ICSI and pregnancy outcome. NOS activity and generation are, however, substrate dependent, thus endogenous arginine synthesis and cellular uptake via cationic amino acid transporter regulate NOS activity. Oral l-arginine supplementation during controlled ovarian hyperstimulation in women undergoing IVF had elevated intrafollicular $NO_2^-/NO_3^-$ concentrations with detrimental consequences on embryo quality, implantations and pregnancy rate. These findings fit well to our own observations showing that elevated l-arginine level in the follicular fluid is associated with less oocytes and embryos. On the other hand, dietary arginine supplementation during early pregnancy enhanced embryonic survival and increased serum l-arginine and NO metabolites in rats. In addition to l-arginine, intrafollicular methylarginines were also found to correlate inversely with oocyte recruitment and fertilization. The synergistic interplay between reactive oxygen species and inflammatory cytokines may contribute to accumulation of methylarginines and may compromise the integrity of l-arginine/NO system. ADMA and MMA inhibit NOS activity and cellular l-arginine uptake, whereas SDMA is a weak inhibitor of cellular l-arginine transport. Interestingly, the activity of enzymes involved in the formation and degradation of ADMA and MMA such as protein methyltransferase (PRMT) and DDAH are regulated in redox- sensitive fashion; oxidative stress enhances the activity of PRMT and inhibits DDAH leading to increased ADMA and MMA concentrations.

It can also be considered that l-arginine and methylarginines are simultaneously released due to the high rate of protein turnover and/or proteolysis during the apoptotic process accompanying oocyte maturation and early embryonic development. This mechanism is likely to be operating because the FF pattern of SDMA which does not undergo enzymatic

degradation proved to be essentially the same as that of ADMA and MMA that are metabolized by DDAH.

The significant positive relationship of arg-MI to the number of IVF oocytes and embryos needs to be commented on. The recently introduced integrated quantification of arginine methylation, arg-MI, is regarded as an independent risk factor for coronary artery disease and for future major cardiac events in stable patients undergoing cardiac evaluation. The reduction of arg-MI may be indicative therefore, of defective global methylation. The significant positive correlation between arg-MI and the reproductive markers suggest a role for methylarginines independent of NOS inhibition and NO generation. Although arg-MI only quantitates arginine methylation, the methyl groups for this process are provided by the common folate-dependent homocysteine/methyonine cycle.

Combined, the determination of l-arginine and its methylated metabolites – ADMA, SDMA, MMA – in the follicular fluid of patients undergoing IVF may be of clinical importance in predicting oocyte quality and maturation, early embryonic development and pregnancy outcome. Further studies are to be conducted to better define the involvement of methylarginines in reproductive performance during IVF.

## *6. Summary of novel results*

6.1. With the increase in our pathophysiological knowledge the options for preventing ovarian hyperstimulation syndrome are increasing, however it is important that only competent professional with sufficient experience in this filed perform ovulation induction treatments. With the random study performed at our clinic we have proved that low dose aspirin is effective in the prevention of OHSS and in easing the symptoms.

6.2. We proved the presence of PACAP in human follicular fluid. This confirms our hypothesis that PACAP plays an important role in follicular fluid as a culture medium for developing oocytes.

6.3. We were the first to write about the connection between the concentration levels of PACAP and the response of the oocytes to gonadotropins, which hints at a possible

physiological relationship. These results turn our attention to the pathomechanism of ovarian hyperstimulation syndrome (OHSS), since higher levels of PACAP concentration in follicular fluid could be an indicator when it comes to the maturation of follicles, while low concentrations of PACAP indicates a risk of developing OHSS.

6.4. With our study we have proved that the follicular fluid of women undergoing IVF treatment contain the most important elements of the l-arginine/NO system, the increased activity of this system has a beneficial effect on the outcome of reproduction. This connection is clinically relevant, since increased levels of l-arginine and methylarginine result in lower levels of oocytes and number of embryos, and previously unknown.

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