

Endometriosis, oxidative stress, clinical implications

Ph.D Thesis Booklet

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1. Introduction

Infertility, which has become an everyday problem in recent decades, forms a significant part of obstetrics and gynecology medicine today. We speak of infertility if a couple trying to have a child fails to conceive after a year of trying. About 15 percent of couples are infertile, and it is also known that in about 40 percent of infertile couples, the cause is to be found in the female member of the couple. According to current statistics, endometriosis can be detected in about 25-50 percent of infertile women. We speak of endometriosis when the endometrium is found outside the uterine cavity. Depending on its occurrence, it can be located in the wall of the uterus (adenomyosis), in the abdominal cavity (ovary, peritoneum, bladder, intestines, etc.), or less often in more distant organs. In the case of deeply infiltrating endometriosis, the depth extension exceeds 5 mm in prominent organs. Endometriosis mainly occurs during the reproductive age, but can also occur during pre-menarche and menopause, and can be associated with severe, chronic pain and subfertility/infertility. It affects around 190 million women worldwide, and in Hungary this figure can be put at 200,000. Regarding its frequency, according to 2010 data, it occurs in about 6-10 percent of women (nowadays, this rate is about 5-6 percent due to population growth). Its prevalence in fertile women of childbearing age is considered to be 0.5-5 percent, while in the case of infertile women it is 25-50 percent. The increasing frequency of the above lesions, or more precisely the increasing frequency of their recognition, is due to the fact that with the spread of our knowledge about endometriosis and the spread of our diagnostic procedures, more and more endometriotic lesions are being recognized. Our clinic has been operating as an endometriosis center for more than ten years, which aims to recognize and treat the disease as soon as possible. Surgery is indicated by the two main symptoms caused by endometriosis, pain and infertility. As a result of cooperation with surgeons and urologists, a multidisciplinary team provides surgical care for patients with endometriosis. However, in patients with endometriosis, despite successful surgical treatment, it is often necessary to use an in vitro fertilization procedure. Our goal was to somehow improve the probability of pregnancy in the case of women suffering from endometriosis, as well as to map out the role of endometriosis in many areas, which is wide-ranging (pain, infertility, cancer, surgical technique) and which determines the quality of life.

1.1 Etiology and pathophysiology of endometriosis

The etiology of endometriosis is still not clearly clarified, and there are several theories regarding its development. Changed lifestyle, lack of exercise, smoking are classified as risk factors, but studies so far have not been able to show a clear correlation with the above.

Transplantation theory:

The most accepted theory is that retrograde menstruation is the most important factor in the development of endometriosis. The theory is attributed to Sampson (John A. Sampson, 1873-1946, American gynecologist), who in 1927 derived endometriosis from the retrograde flow of menstrual blood through the uterine horns, its consequent peritoneal dissemination and peritoneal implantation.

Immunological theory/dysfunction:

Since an inflammatory reaction can be detected during the development of endometriosis (the role of oxidative stress and inflammation), the adhesion of endometriosis implants can also be caused by a defective immune response.

Metaplastic theory:

The metaplastic theory refers to the metaplasia of remnants of the coelom epithelium or Müllerian duct.

Angiogenic, lymphogenic spread:

The spread of endometrial parts via lymphatic channels and blood vessels was first described by J. Halban (1925), who also observed endometriosis foci in lymph nodes and lungs.

Micro-traumatization-reparation theory:

Leyendecker derives endometriosis and adenomyosis from the micro-traumatization and subsequent tissue repair (TIAR, tissue injury and repair) that occurs during uterine peristalsis, during which fragments of the basal endometrium enter the abdominal cavity and the deeper myometrium.

Embryonic remnant theory:

According to Sasson, during embryogenesis some of the endometrial cells are transferred to the abdominal cavity instead of the uterus, and these cells can be activated later after puberty under the influence of estrogen and progesterone.

Environmental effects:

The role of environmental influences was also described, including the role of changed lifestyles and dioxin. (Changed circadian estrogen levels due to night shift work is associated with a 50 percent increase in the risk of endometriosis.)

Endocrine aspect:

Endometriosis is characterized by progesterone deficiency or resistance, but at the same time, increased sensitivity to the effect of estrogen can be observed. Changes in apoptotic processes, the presence of oxidative stress, and changes in the cell cycle were described in the granulosa cells surrounding the oocyte.

Genetic factor:

The genetic background of endometriosis is an active part of research, however, it is known that in families affected by endometriosis there is a 5x higher frequency of occurrence than in first degree relatives.

Iatrogenic route/extragenital endometriosis:

The implantation theory can be used to explain the so-called iatrogenic endometriosis, which means endometriosis that occurs in surgical scars or mucosal and tissue injuries after gynecological interventions.

Other etiological factors:

The background of endometriosis in phenotypically female patients with gonadal dysgenesis and in men is exogenous estrogen intake.

All in all, we can say that all forms of endometriosis are not explained by any currently known etiological factor.

1.2 Types of endometriosis

Depending on the location of the ectopic mucosal islands, the different manifestations are external genital endometriosis, internal genital endometriosis (adenomyosis uteri) and deep infiltrating endometriosis (DIE). In the case of deeply infiltrating endometriosis, another classification is based on the ENZIAN classification, while in the case of peritoneal, ovarian and fallopian tube endometriosis, the rAFS (revised American Fertility Society) score is used. In both cases, the classification is based on the location and size of the lesion, however, the resulting point system does not correlate with the patients' complaints caused by endometriosis. Due to the above and the lack of therapeutic consistency, the classification according to the classifications is not widely used in everyday practice. According to the grouping we also use, which is the most common in practice, its four main manifestations are adenomyosis, peritoneal endometriosis, ovarian endometriosis, and deep infiltrating endometriosis.

1.3 Symptoms of endometriosis and infertility

The symptoms caused by endometriosis can be divided into two large groups. One is the pain it causes, and the other is infertility, the frequency of which among endometriosis patients can be estimated at around 30-50 percent. Infertility can be caused by distorted pelvic anatomy caused by endometriosis, altered peritoneal and endometrial functions detected and previously described in the background of the disease, as well as disorders of the endocrine system and ovulation. Proceeding along the main pillars of endometriosis causing infertility, we looked for correlations at the cellular level by examining the serum, follicular fluid, and the nutrient solution surrounding the embryo, searching for biomarkers whose role can be demonstrated in the creation of infertility. In addition to the cellular level tests, we also planned to study the effect of endometriosis on causing significant organ damage.

Based on the above, we initially looked for possible correlations between serum and nutrient solution samples obtained during reproductive procedures and endometriosis.

2. Oxidative stress and endometriosis

Oxidative stress, which plays a role in the development of endometriosis, is an etiological factor increasingly attracting attention. Oxidants (reactive oxygen species, ROS) are unstable molecules that attack any chemical substance they come into contact with. Macromolecules in their path are modified both structurally and functionally through lipid peroxidation, DNA damage and apoptosis. Depending on the tissue concentration, they can have a useful physiological effect (e.g. in fertilization) or they can cause pathological damage to cell components (e.g. lipids, proteins). Oxidants are actually free radicals in everyday usage, which play a role in the development of many diseases – including inflammatory and tumor diseases. However, the human body has a defense mechanism against oxidants: these are antioxidants. Two forms of defense mechanisms are known: enzymatic and non-enzymatic defense pathways. The balance of free radicals and the antioxidants that fight against them is essential for the physiological functioning of the body and the prevention of the development of diseases. However, when the delicate balance of free radicals and antioxidants is disturbed, i.e. free radicals exceed the 'cleaning' capacity of antioxidants, oxidative stress is created. The role of oxidative stress in fertilization is of great importance, which is dealt with in several literary publications. Oxidative stress causes damage to the sperm, egg and embryo. It can affect ovulation, fertilization, embryo development and implantation. (Presumably, it has a

role in both natural and in vitro fertilization.) Oxidants are attributed a role not only in reproductive processes, but also in the development of endometriosis. It is a known fact that the follicular fluid contains a high number of antioxidants, which protect the oocyte from ROS-induced damage. Since endometriosis was the focus of our research, the question arose as to how the proportion of antioxidants in female patients suffering from endometriosis and undergoing infertility treatment is changing. The presence of oxidative stress is known in connection with endometriosis, but does this effect appear in connection with the in vitro fertilization procedure?

2.1 Our objective

In the course of our studies, we set out to map whether female patients suffering from endometriosis undergoing in vitro fertilization have differences in antioxidants compared to female patients not suffering from endometriosis. For this, we used the total antioxidant capacity (TAC) and another antioxidant molecule, 8-hydroxy-2'-deoxyguanosine (whose intracellular concentration is also a measure of oxidative stress) as a basis.

2.2 Patient selection and sampling

Our research included 61 patients between May 1 and June 1, 2016, and February 1 and April 1, 2018, who took part in an in vitro fertilization treatment at the Reproduction Center of the Obstetrics and Gynecology Clinic of the University of Pécs. Female patients suffering from endometriosis (and therefore at high risk for oxidative stress) and those not affected by it were examined and evaluated in a separate group. Endometriosis was classified according to the rAFS (revised American Fertility Society) stages; accordingly, stage II-IV patients (II-mild, III-moderately severe, IV-severe) were included in the research. The patients selected in this way underwent general clinical examinations before the superovulation treatment (cervical cancer screening, serum hormone level measurements, viral serologies, evaluation of the shape of the uterus, andrological examination of the male partner). The sampling included blood sampling between days 3 and 5 of the menstrual cycle before the stimulated cycle, and on the morning of the follicle puncture day, as well as aspiration of follicular fluid 36 hours after ovulation induction under transvaginal ultrasound guidance. During the procedure, the patients received a long or short protocol administration of the gonadotropin releasing hormone (GnRH) agonist (triptorelin), and the stimulation was done with individual doses of

recombinant follicle stimulating hormone (rFSH). The maturation of the follicles was checked by transvaginal ultrasound every other day from the sixth day of the cycle. If the size of two follicles exceeded 17 mm, ovulation induction followed, in the form of human choriogonadotropin injection (hCG). Follicular fluid was aspirated under intravenous sedation 36 hours after ovulation induction under transvaginal ultrasound control. The oocytes obtained in this way were placed in G-MOPS™ medium (Vitrolife®, Göteborg, Sweden), and after appropriate centrifugation and freezing, they were stored at -80 °C until the tests.

2.3 Fertilization procedure and measurement methods

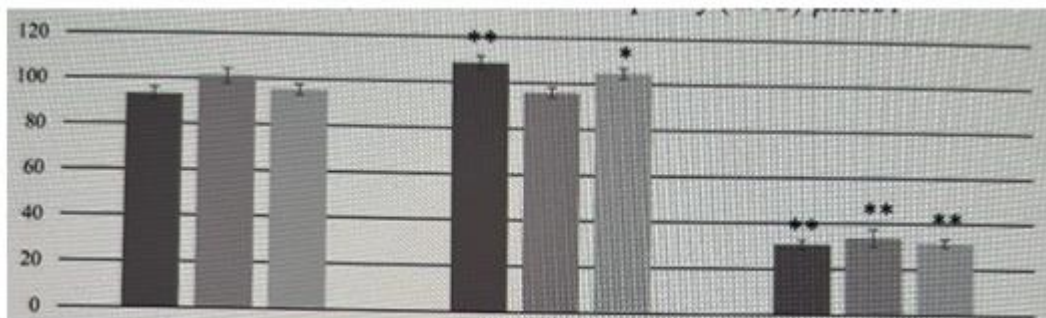
The fertilization procedure was carried out by intracytoplasmic sperm injection (ICSI) depending on andrological status (in the case of a sperm count below 15 M/ml), maternal age (>35 years) and the number of previous IVF cycles (>2). Oocytes from patients who did not meet the above conditions were subjected to a conventional in vitro fertilization procedure, and after being in a bicarbonate-buffered medium for 24 hours, they were placed in the G-I™ v5 medium also used during the ICSI procedure (Vitrolife®, Göteborg, Sweden). The embryo transfer took place 3-5 days after the egg retrieval. According to the wishes of the patients, one, two or three embryos were implanted. As an evaluation of the success of the procedure, 21 days after the embryo transfer, a vaginal ultrasound examination was performed to assess the ovaries. The laboratory measurements were performed with chemiluminescence assay for total antioxidant capacity (TAC) and enzyme-linked immunosorbent assay (ELISA) for 8-hydroxy-2'-deoxyguanosine (8-OHdG) at the Institute of Laboratory Medicine of the University of Pécs. Statistical analysis was performed using SPSS 21.0.

2.4 Results

The role of reactive oxygen radicals in female reproductive processes is still unclear. It is certain that not only the individually determined level of reactive oxygen radicals and antioxidants, but also the balance they create is extremely important. Therefore, it is not easy to determine the normal serum level of total antioxidant capacity, determined in our laboratory for healthy adults; its level was determined to be between 119.3 and 134.4 µmol/l (95 percent confidence level) measured against the Trolox equivalent (TE, a water-soluble vitamin E analog) as a standard. We used this total antioxidant capacity interval as a basis for our tests. The basic total antioxidant capacity level (TE, 95.44 ± 17.79 µmol/l) measured in the

cycle before the stimulation increased significantly ($104.78 \pm 17.42 \mu\text{mol/l}$) on the day of the follicular fluid aspiration as a result of the controlled ovarian stimulation. Compared to the TAC level of the serum, the TAC level of the follicular fluid decreased markedly ($31.62 \pm 13.56 \mu\text{mol/l}$). A completely different response was shown in relation to 8-hydroxy-2'-deoxyguanosine: controlled hyperstimulation resulted in a significant decrease in serum 8-OHdG (compared to the serum value of $17.61 \pm 5.15 \text{ ng/ml}$ measured on day 21 of the unstimulated cycle, it decreased to $15.61 \pm 5.26 \text{ ng/ml}$ measured on the day of aspiration), then decreased further in the follicular fluid (to $11.10 \pm 2.85 \text{ ng/ml}$), although to a much lesser extent than for the follicular fluid TAC level. The serum and follicular fluid total antioxidant capacity (TAC) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels are summarized in the figure below.

Total non-enzymatic antioxidant capacity



Maternal serum on day 21

Maternal serum on the day of follicular fluid aspiration

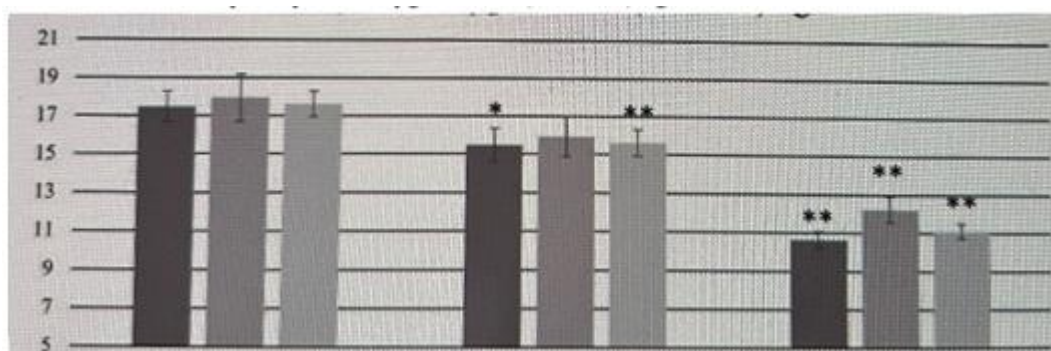
Follicular fluid

Patients without endometriosis (No=43)

Patients with endometriosis (No=18)

All patients (No=61)

8-hydroxy-2'-deoxyguanosine (8-OHdG) ng/ml



Maternal serum on day 21

Maternal serum on the day of follicular fluid aspiration

Follicular fluid

Patients without endometriosis (No=43)

Patients with endometriosis (No=18)

All patients (No=61)

Image/Values of total antioxidant capacity (TAC) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) measured in serum and follicular fluid in female patients who participated in an in vitro fertilization procedure

The test values of patients with endometriosis were evaluated in a separate group and compared with the other non-endometriosis samples. The serum TAC value of female endometriosis patients before stimulation decreased from $101.23 \pm 13.74 \mu$ to $95.94 \pm 9.36 \mu\text{mol/l}$ after controlled hyperstimulation, and further decreased to $33.64 \pm 16.79 \mu\text{mol/l}$ in the follicular fluid. The corresponding TAC values in female patients without endometriosis increased from $92.97 \pm 18.88 \mu\text{mol/l}$ to $108.57 \pm 18.74 \mu\text{mol/l}$ in the serum, and then decreased to $30.77 \pm 12.64 \mu\text{mol/l}$ in the follicular fluid. The difference between the TAC serum level after stimulation and the TAC level of the follicular fluid in the two groups proved to be significant ($p < 0.001$ and $p < 0.013$). Regarding the level of 8-hydroxy-2'-deoxyguanosine, no difference was observed between the two groups in serum levels before ($17.47 \pm 5.17 \text{ ng/ml}$ and $17.94 \pm 5.22 \text{ ng/ml}$) and after ($15.47 \pm 5.62 \text{ ng/ml}$ and $15.93 \pm 4.43 \text{ ng/ml}$) stimulation; at the same time, the level measured in the follicular fluid was higher in the non-endometriosis group than in the endometriosis group ($10.63 \pm 2.74 \text{ ng/ml}$ in the endometriosis group and $12.22 \pm 2.88 \text{ ng/ml}$ in the non-endometriosis group, $p < 0.047$). If 8-OHdG is functionally assigned to the group of TAC, a clear difference was visible between the measured values of these two biomarkers, since 8-OHdG in the follicular fluid proved to be independent of changes in TAC. Overall, however, their cumulative serum levels were inversely proportional. The curiosity about where the above oxidative stress markers can be derived from resulted in an additional (supplementary) investigation. For this purpose, the ratio of total antioxidant capacity to albumin and the ratio of 8-hydroxy-2'-deoxyguanosine to albumin were determined simultaneously in serum and follicular fluid in 30 patients. The ratio of TAC to albumin was about three times higher in the serum than in the follicular fluid (2.29 ± 0.33 versus 0.69 ± 0.14 , $p < 0.001$), while the ratio of 8-OHdG to albumin remained practically at the same level (0.31 ± 0.09 in the serum and 0.27 ± 0.07 in the follicular fluid, $p < 0.026$). The above albumin concentration in the serum was $43.52 \pm 2.88 \text{ g/l}$, and in the follicular fluid $37.87 \pm 3.90 \text{ g/l}$. Based on these, it can be assumed that the majority of 8-OHdG measured in the follicular fluid is plasma filtrate, while the TAC measured in the follicular fluid is produced locally in the ovaries. In our studies, the values measured in the serum were not, but the TAC and 8-OHdG values measured in the follicular fluid were inversely proportional to the number of embryos evaluated as 'good quality'. In contrast, in the

subgroups with and without endometriosis, neither serum nor follicular fluid TAC and 8-OHdG levels showed a significant correlation with mature oocytes and high-quality embryos. Contrary to our expectations, we measured significantly higher 8-OHdG serum levels in female patients with endometriosis who had a clinical pregnancy compared to those who did not (18.14 ± 4.26 ng/ml and 13.71 ± 3.54 ng/ml, $p < 0.029$). Although a role is attributed to oxidative stress in the pathophysiology of endometriosis, we would have expected a higher level of 8-OHdG as a consequence of the DNA damage caused by it, which, as previously written, is an indicator of DNA damage.

Of the 61 patients included in our study (18 with endometriosis and 43 without endometriosis), clinical pregnancies occurred in 22 cases, of which 9 patients with endometriosis (9 pregnancies out of 18 patients, which is 50 percent) and 13 patients without endometriosis (13 out of 43 patients, which corresponds to 30.23 percent). Serum and follicular fluid TAC levels of non-endometriotic patients with clinical pregnancy were significantly higher than those who did not develop a clinical pregnancy (117.10 ± 17.46 $\mu\text{mol/l}$ against 104.74 ± 18.30 $\mu\text{mol/l}$ and 38.39 ± 18.81 $\mu\text{mol/l}$ against 27.66 ± 7.73 $\mu\text{mol/l}$). For the former, it was $p < 0.047$, for the latter, $p < 0.016$. In contrast, there was no apparent difference in serum and follicular fluid 8-OHdG levels in the pregnant and non-pregnant, non-endometriosis groups.

Our research proved that the two oxidative stress markers, TAC and 8-OHdG, do not change together in patients receiving in vitro fertilization. In contrast to TAC, 8-OHdG is mostly derived from circulating plasma rather than local production and is a less sensitive indicator of reproductive potential than TAC. However, due to the relatively small number of cases, the results should be treated with sufficient caution, and further, extended studies are necessary for their certainty.

When investigating the molecular background of endometriosis and infertility, in addition to examining the effect of oxidative stress, we searched for an additional mechanism, a molecule, through which the fertility chances of patients living with endometriosis are worse, and if identified, the chances of pregnancy of female patients with endometriosis who participate in the in vitro fertilization procedure could improve. For this purpose, unlike the previous ones (serum and follicular fluid testing), we planned to test the embryo nutrient solution used during the in vitro fertilization procedure.

3. Haptoglobin and endometriosis

In order to increase the success rate, in 2013 Montskó and his colleagues searched for a molecule that could be used to determine the viability of the embryo using a non-invasive method. During their research, they examined the embryo nutrient solution used in connection with the IVF procedure. As a result of all this, through the applied molecular cell biology tests (liquid chromatography and mass spectrometry), a protein - namely the alpha-1 (α -1) haptoglobin fragment - was identified, which was suitable for judging non-viable embryos in the tested samples. The higher the amount of the specified protein in the sample of the given nutrient solution, the less likely the embryo became viable.

3.1 Objectives

Knowing the above, focusing on endometriosis, our idea was to increase the fertility chances of patients suffering from endometriosis, with the help of determining the alpha-1 haptoglobin fragment level of the embryo nutrient solution. The protein is already contained in the nutrient solution (as part of the albumin added to the nutrient solution), its amount changes during the development of the embryo, depending on the outcome of the implantation. Thus, by determining its quantity, those embryos (on the basis of nutrient solutions) which may be suitable for pregnancy on the basis of the concentration (in the case of the lowest value) could be selected. The aim of the above research was to evaluate the quality of the embryos not only based on the existing morphological features, but to supplement it with the quantitative determination of the alpha-1 haptoglobin fragment, to increase the success of artificial insemination treatments.

3.2 Selection of patients and methods

In the investigated period between October 2014 and September 2016, under the technical and personal conditions at our disposal, we had the opportunity to analyze a total of 30 samples of nutrient solutions from 16 infertile female patients suffering from endometriosis in laboratory conditions, while in the case of non-endometriosis patients this number was a total of 110 samples from 60 patients. In the endometriosis group, we had the opportunity to process 1 sample from 3 patients, 2 samples from 12 patients, and 3 samples from 1 patient. In the non-endometriosis group, we examined 1 sample each of 19 patients, 2 samples each of 33 patients, 3 samples of 7 patients, and 4 samples of 1 patient. We have separately indicated the reason for the IVF treatment, the type of endometriosis, its existence, the method of diagnosis,

as well as whether surgical intervention was performed in connection with endometriosis, and if so, what type. In addition to the underlying disease, the cause of infertility in patients with endometriosis was the origin of the horn (2 patients) and the male factor (3 patients). In the non-endometriosis group, 14 cases (23.3 percent) caused infertility, 25 cases (41.6 percent) had a male factor as the diagnosis, 1 patient (1.6 percent) had a myoma, and 20 (33.3 percent) had an unknown cause of infertility. In our research, we examined the concentration of the alpha-1 haptoglobin fragment in the above two groups, endometriosis and non-endometriosis. The concentration was determined by liquid chromatography and mass spectrometry in ng/25 μ l. During the tests, the amount of the haptoglobin fragment was compared to the alpha-1 haptoglobin fragment concentration of the blank nutrient solution. During the follow-up of the patients, we kept an eye on whether a pregnancy occurred during the given IVF procedure, and if so, with what outcome.

3.3 Our results

Of the 16 patients with endometriosis included in our study, 10 women became pregnant. The 10 pregnancies ended in childbirth in 6 cases, two of which gave birth to twin newborns. In 2 cases, the fate of the pregnancy ended with a missed abortion (dead pregnancy, incomplete abortion), in 2 cases we have no knowledge of its development. In the case of all nutrient solutions, the amount of the alpha-1 haptoglobin fragment was determined, which was determined in ng/25 μ l. In the case of patients suffering from endometriosis, the average alpha-1 haptoglobin fragment level of the embryo-nutritional solutions resulting in pregnancy was 38.68 ng/25 μ l, in contrast, in patients who did not develop pregnancy, this figure was 46.57 ng/25 μ l. In the case of non-viable embryos, a higher alpha-1 haptoglobin fragment level was confirmed in this group. We also determined the average alpha-1 haptoglobin fragment in the nutrient solutions of the patients whose pregnancy ended in delivery, here we obtained 39.82 ng/25 μ l. Based on the previous concept, we would have expected a lower alpha-1 haptoglobin fragment value in the case of the delivered patients, however, due to the small number of cases, it is not possible to draw a conclusion in this regard. Out of 60 patients not affected by endometriosis, pregnancy developed in 23 cases. The 23 pregnancies ended in childbirth in 11 cases, and two of them (also in the endometriosis group) gave birth to twin newborns. The fate of the pregnancy in this group ended with a missed abortion in 3 cases, and in 9 cases we had no knowledge of the further fate of the pregnancy. Based on the above data, the pregnancy rate for patients without endometriosis turned out to be 38.33 percent,

however, due to the different number of samples per patient (1, 2, 3, and 4 samples), the pregnancy rate for all samples is 22.72 percent. In the case of all nutrient solutions, the amount of the alpha-1 haptoglobin fragment was determined, which was also determined in ng/25 µl. In the case of patients without endometriosis, the average level of alpha-1 haptoglobin fragment in embryo-nutritional solutions that ended in pregnancy was 38.60 ng/25 µl, on the other hand, in patients who did not develop pregnancy, this figure was 43.41 ng/25 µl. In this group, we also determined the average alpha-1 haptoglobin fragment in the nutrient solutions of the patients whose pregnancy ended in delivery, and here we obtained a result of 36.59 ng/25 µl. In the case of non-viable embryos, a higher alpha-1 haptoglobin fragment level was also observed in this group. Our calculated and measured results are summarized in the table below.

| | ENDOMETRIOSIS | NO ENDOMETRIOSIS |
|---|--------------------|--------------------|
| number of patients (n) | 16 | 60 |
| number of samples (n) | 30 | 110 |
| number of pregnancies (n) | 10 | 23 |
| pregnancy rate for patients (%) | 62.5 | 38.33 |
| pregnancy rate for sample number (%) | 40 | 22.72 |
| number of births (n) | 6 (2 gemini) | 11 (2 gemini) |
| number of missed ab. (n) | 2 | 3 |
| fate of pregnancy unknown (n) | 2 | 9 (1 gemini) |
| α-1 haptoglobin fragment mean±SD in pregnant samples (ng/25 µl) | 38.68±8.214 | 38.60±8.524 |
| α-1 haptoglobin fragment mean±SD in born samples (ng/25 µL) | 39.82±8.369 | 36.59±7.008 |

| | | |
|---|-----------------------------------|-----------------------------------|
| α -1 haptoglobin fragment mean \pm SD in non-pregnant samples (ng/25 μ l) | 46.57\pm7.025 | 43.41\pm9.075 |
|---|-----------------------------------|-----------------------------------|

Table/summarization of results for endometriosis and non-endometriosis patients

The measured alpha-1 haptoglobin fragment values were subjected to statistical analysis, using the ANOVA analysis of variance. During the analysis, we could not show a significant difference in the alpha-1 haptoglobin fragment level measured in the in vitro fertilization nutrient solution samples collected from infertile female patients affected by endometriosis and those without it (p=0.393). According to our assumption, the correlation observed between the haptoglobin fragment and the development of pregnancy is not influenced by the presence of endometriosis. However, a significant difference in the alpha-1 haptoglobin fragment levels was confirmed – in the samples of both endometriosis and non-endometriosis patients – in the pregnant and non-pregnant groups (p=0.001). In the case of viable embryos, the alpha-1 haptoglobin fragment therefore showed a lower value than in the case of non-viable embryos, thereby confirming the previous statement that the alpha-1 haptoglobin fragment ratio measured in the nutrient solution is inversely proportional to the viability of the embryo.

Since the tests with regard to the nutrient solution biomarker and endometriosis that we investigated did not produce the expected results, we started our research to examine another effect of endometriosis on fertility. We then turned our attention to significant pelvic anatomical damage caused by endometriosis.

4. Surgical aspects of endometriosis

The effect of endometriosis causing distorted pelvic anatomy is often encountered in everyday practice. This can appear in the form of fallopian tubes blocked by the disease, or as a result of scars created during the repair of sterile inflammation due to endometriosis, which result in the blurring and adhesion of normal organ boundaries. In both cases, the development of a possible pregnancy becomes hindered. Although the professional position/proposal for this is constantly changing, according to our current knowledge, the gold standard for the treatment

of endometriosis is still surgical treatment in properly selected cases. Fertility chances can be improved by excision of the endometriosis changes of the affected organs and the resulting scars. The Obstetrics and Gynecology Clinic of the University of Pécs has been operating as an endometriosis center providing care for deeply infiltrating endometriosis for more than ten years. After March 1, 2021, two independent teams were established, with the participation of two gynecologists and two surgeons (Pécs University Clinical Center Surgical Clinic).

Gynecologist Dr. Tamás Varga and surgeon Dr. József Baracs work in one team, surgeon Dr. Ágoston Ember and myself work in the other team. In addition, for about 4 years, a special appointment has been provided for patients suffering from endometriosis, where the two gynecologists involved in surgical care carry out examinations, with additional surgical and urological examinations if necessary. Dr. Tamás Varga and I performed several surgeries between 03.01.2021 and 12.01.2023 due to the diagnosis of endometriosis: 84 peritoneal endometriosis excisions, 66 interventions due to ovarian endometriosis, 65 deeply infiltrating endometriosis excisions (including 4 bladder, 9 vagina and one mesorectum node resections), 1 compartment peritoneum endometriosis removal, and 36 colon endometriosis removals were performed.

Among the above, I would highlight the surgical treatment of endometriosis, which is often seriously challenging and affects the intestinal wall. Intestinal involvement occurs in approximately 8-12 percent of endometriosis patients, with approximately 90 percent affecting the sigmoid colon or rectum. It is usually present together with deeply infiltrating endometriosis, and plays a role in both pelvic pain and infertility. The effect of intestinal endometriosis on fertility is not yet clear, so the optimal management of patients with a desire to have children is not clear. According to the 2022 endometriosis guideline of the ESHRE (European Society of Human Reproduction and Embryology), surgical treatment for colorectal endometriosis has a good effect on fertility. Based on the above, at our clinic in the above period, we decided to remove intestinal endometriosis in 36 cases. In accordance with the ESHRE guideline, the interventions were carried out by a multidisciplinary team, using a minimally invasive technique and radical excision. In 3 of the 36 cases, the endometriosis nodule was removed with a shaving technique, in 16 cases with a discoid resection technique (in one case both the resection and the suture were performed without the use of a sewing machine), and in 17 cases with a segment resection. During the segment resections, we used the NOSE (Natural Orifice Specimen Extraction) technique in selected cases, trans-anally. Given that the indication for surgery was mainly not due to a desire for fertilization, we have little data regarding the pregnancies conceived in the colorectal endometriosis group:

spontaneous pregnancy occurred in two patients after the surgical intervention, in both cases the pregnancy ended in delivery. (One of them had 3 unsuccessful in vitro fertilizations before the operation.) Two patients recently underwent IVF implantation at our clinic (in their case, due to the severity of the disease, we recommended IVF from the start), 1 patient is undergoing ovarian stimulation, and unfortunately one of them has had 3 unsuccessful IVFs since the operation. The surgical solution of deeply infiltrating endometriosis is an effective solution available at centers, but it has a significant complication rate, especially if there is bowel involvement. According to our current data (2022 ESHRE guideline), the total intraoperative complication rate is 2.1 percent, and the total postoperative complication rate is 13.9 percent, of which 9.5 percent are minor complications and 4.6 percent are major complications. According to a 2022 study, the rate of temporary stoma placement necessary during colorectal endometriosis surgeries is 2.7 percent, which is necessary prophylactically in the event of an intraoperative complication or a very low resection. Due to the complications surrounding the stoma, efforts are being made to reduce the number of prophylactic stomas worldwide. 3.07 percent of the deep infiltrating endometriosis surgeries performed at our Clinic in these two and a half years were performed through open surgery, the remaining 96.93 percent through minimally invasive, laparoscopic surgery. The total intraoperative complication rate was found to be 3.07 percent, which means 2 complications. In one patient, a load-relieving transversostomy became necessary during the operation due to the low distal plane of the resection (this represents 2.77 percent of the intraoperative stoma), in the other case, an intraoperative iatrogenic ureteral injury was confirmed, for which it was sutured. During our operations, our permanent urologist is Dr. Dániel Bányai from the PTE KK Urology Clinic, who participates in the examination, surgical solution and follow-up in all cases of urological involvement. Postoperative complications occurred in 4 cases (6.15 percent). In two patients, insufficiency of the intestinal suture was confirmed, which necessitated the placement of a temporary stoma in one case, and in one case segment resection was performed via laparotomy after the primary laparoscopic discoid resection. In one case, a unilateral ureteral injury was confirmed in the background of postoperative urination, which was resolved without reoperation with the placement of a DJ stent. In one case, the ureteral injury, detected as an intraoperative complication and sutured, necessitated repeated laparoscopy and ureteral re-suturing on the day after the operation due to suture insufficiency. The rate of ureteral injuries in our examined group was 3.07 percent. In both cases described above, the transient stoma was closed 4 months after placement.

The uterine-ureteral crossing of the sacro-uterine ligament-artery, which is a typical site of deeply infiltrating endometriosis, and its surroundings pose a significant threat to the urological organs. In the case of advanced endometriosis, the compression of the nodule and scar tissue on the ureter can cause difficulty in the outflow of urine, with consequent retrograde ureter dilation and renal pelvis dilation. Unrecognized cases can even cause irreversible kidney damage over the years. During the above period, in one case, the known enlargement of the renal pelvis proved to be irreversible despite the removal of the scar tissue that caused it, a nephrectomy was performed one year after the operation, and at the beginning of this year, in two cases, cysts formed as a result of pelvic endometriosis were recognized, which also led to nephrectomy. Out of the 65 patients who underwent surgery due to deep infiltrating endometriosis, we were unable to perform the planned intervention in two cases (in one case due to extensive involvement of the rectovaginal septum and a very low rectal nodule, in one case due to the inflammation seen during the operation and due to fistulization of the endometrioma towards the vagina). Although endometriosis is a benign entity, it can be seen from the above data that it can have serious consequences, which can significantly affect the quality of life and fertility. The danger lies not only in these, but also in the case of a pelvic abscess or, in rare cases, malignancy.

5. Endometriosis and malignancy

The frequency of malignant transformation of endometriosis is currently unknown, however, people suffering from the disease have approx. Over the years, 1 percent develop a neoplasm related to endometriosis. The exact process of malignant transformation is not known, but various hormonal factors, inflammation, family predisposition, genetic modifications, growth factors, nutrition, altered immune system, environmental factors and oxidative stress may play a role in carcinogenesis. Early menarche, low birthrate (parity), late menopause and infertility may also contribute to the pathogenesis of these tumors. It mostly occurs in patients who have previously undergone surgery for endometriosis. The vast majority of the above malignant tumors are of ovarian origin (~78 percent), however extragonadal origin is also known. Malignant neoplasms arising from adenomyosis are a rare entity. It is important to note that the diagnosis in these cases is often delayed due to the negativity of the eutopic endometrium. Malignancies arising from endometriosis are predominantly low-grade tumors confined to the anatomical boundaries of the given organ. The two most common histological forms are

endometrioid (70%) and clear-cell carcinoma (14%). With our case report, we would like to draw attention to the fact that, although it is a very rare entity, in the presence of endometriosis, we must also consider the possibility of malignant transformation.

5.1 Our case

A 73-year-old female patient, who has been in menopause for 23 years, was investigated for uncertain lower abdominal pain and frequent urge to urinate. She had no known medical history of endometriosis. She had two spontaneous deliveries and a tonsillectomy in her anamnesis. Diabetes and obesity were not present. During her clinical examination (bimanual physical examination and vaginal ultrasound), the formula filling the pelvis below the navel was confirmed. With an ultrasound examination, the formula appeared to be multicompartamental, contained growths, presumably of ovarian origin, in addition to a small uterus. Free abdominal fluid was not visible. An elevated CA-125 value (238 U/ml, normal value <30 U/ml) was found during the laboratory examination.

5.2 Surgical care

The patient had a laparotomy at our clinic due to the above: a small uterus was visible with an intact right side and a cystic left adnexum attached to the pelvic wall. During the removal of the left adnexal formula, abundant brown discharge was discharged from it. After that, a hysterectomy and right adnexectomy were performed, along with cytological sampling of free abdominal fluid and flap resection (infracolicus).

5.3 Histological results and postoperative treatment

The right ovary, which did not contain macroscopically pathological parts, the 6.5 cm long uterus and the 9x4.5x7 cm cystic left adnexum were subjected to pathological examination. When the uterus was opened, in addition to the atrophic endometrium (<1 mm), a lesion with a maximum diameter of 2 cm, resembling an adenomyoma, and a typical myoma nodule with a diameter of approximately 2 cm were visible in the myometrium. During the microscopic examination of the area, in addition to adenomyoma details, invasive endometrioid adenocarcinoma foci could be identified according to the location of the adenomyosis. The performed immunohistochemical examination showed positivity for estrogen receptor

(estrogen receptor/ER), progesterone receptor (progesterone receptor/PR), cyclin-dependent kinase inhibitor 2A (p16), tumor protein p53 and MIB1. (The MIB-1 test is used to determine the proliferation index, especially in tumorous cases, to decide whether the change is benign or malignant. Cyclin-dependent kinase inhibitor 2A, or p16, is a tumor suppressor that slows down cell division, and its gene mutation plays an important role in carcinogenesis. p53 is also a protein responsible for regulating the cell cycle and a tumor suppressor at the same time, which is key in suppressing tumor processes.) Macroscopically, the lesion, which looked like a typical myoma nodule, turned out to be a hyalinized leiomyoma. In the case of myomas, hyalinization is a type of degeneration, during which some areas soften and turn into a yellowish, gelatinous mass. No pathological changes could be identified in the right adnexum, however, endometrioid ovarian adenocarcinoma was found in the left ovary on the basis of cystadeno-fibroma. The free abdominal fluid sample taken for the purpose of the pleural effusion and cytological examination did not show malignancy. After the surgical treatment, the onco team committee recommended chemotherapy (Taxol-Carboplatin).

5.4 Discussion

The relationship between adenomyosis and endometrial adenocarcinoma has been investigated in several previous publications. Among patients who have undergone hysterectomy, the co-occurrence of the two pathologies varies between 10 and 70 percent. The development of endometrial tumors is still unclear in patients with adenomyosis. In addition to malignant transformation, cofactors such as long-term (even for years), unbalanced estrogen therapy, inflammation, improper functioning of the immune system, and oxidative stress can also play a role, all of which are attributed a role in carcinogenesis. In contrast to normal endometriotic cells, pronounced p53 expression has been described in atypical endometriotic cells. In the case we present, a low-grade tumor with estrogen receptor (ER+), progesterone receptor (PR+), p16, p53 and MIB-1 positivity was detected without any hormonal history. The patient had no history of endometriosis (or similar complaints). Although in the majority of malignant processes caused by endometriosis, anamnestic, long-term hormone therapy (mostly estrogen monotherapy) can be found, a clear cause and effect relationship between hormone replacement and carcinogenesis has not been known until now. In the case of the patient described by us, not only was a uterine tumor on the basis of adenomyosis confirmed, but also a malignant tumor of epithelial origin affecting the left

ovary (adenocarcinoma endometrioides ovarii on the basis of cystadeno-fibroma), which does not show a connection with endometriosis. The co-occurrence of the above two independently developed malignant neoplasms is unusual. Due to the rarity of such cases, we would consider it very important to collect and publish all data for all similar cases, thus providing the opportunity to better understand the development of this type of tumor. Getting to know and understanding this would make it possible to introduce a screening test, with the help of which even cases without complaints could be recognized as soon as possible. Based on this knowledge, it would also be important to make therapeutic recommendations that could make the treatment of malignant tumors specifically caused by adenomyosis more effective.

6. New results

- During our research, the total antioxidant capacity and 8-hydroxy-2'-deoxyguanosine values measured in the follicular fluid, known as oxidative stress markers, were inversely proportional to the number of embryos evaluated as 'good quality'.
- In the subgroups with and without endometriosis, neither serum nor follicular fluid total antioxidant capacity and 8-hydroxy-2'-deoxyguanosine levels showed a significant correlation with mature oocytes and high-quality embryos.
- We could not detect any significant difference in the haptoglobin alpha-1 fragment level measured during our tests in the samples of in vitro fertilization nutrient solution processed from infertile female patients affected by endometriosis and those without it.
- A significant difference in the haptoglobin alpha-1 levels in the pregnant and non-pregnant groups was confirmed in the embryo nutrient solution samples, confirming a previous research result.
- Between 03.01.2021 and 12.01.2023, the intraoperative complication rate of all surgical procedures performed at our clinic due to deep infiltrating endometriosis (65) was 3.07 percent, and the postoperative complication rate was 6.15 percent.
- The malignant lesion presented in our case, which did not cause any complaints, developed on the basis of adenomyosis, draws attention to the need to introduce a screening test.

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LIST OF SCIENTIFIC PUBLICATIONS, LECTURES

Scientific publications published on the topic of the dissertation

1. Ákos Várnagy, Tamás Kószegi, Erzséber Györgyi, **Sarolta Szegedi**, Endre Sulyok, Viktória Prémusz & József Bódis (2018)
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Human Fertility, DOI: 10.1080/14647273.2018.1535719

2. **Szegedi S**, Koppan M, Varga T, Kovacs K, Tinneberg HR, Bodis J.
Endometrioid adenocarcinoma arising from adenomyosis: a case report.
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3. Bohonyi N, Pohóczky K, Szalontai B, Perkecz A, Kovács K, Kajtár B, Orbán L, Varga T, **Szegedi S**, Bódis J, Helyes Z, Koppán M.
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4. **Szegedi, Sarolta** és Várnagy, Ákos és Bódis, József (2013)
Humán choriogonadotropin (hCG), az „everything molecule” [Human choriogonadotropin (hCG), the “everything molecule”]
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5. Matyas Meggyes^{1,2}, Laszlo Szereday^{1,2}, Noemi Bohonyi³, Miklos Koppan³, **Sarolta Szegedi**³, Anna Marics-Kutas³, Mirjam Marton¹, Anett Totsimon¹, Beata Polgar^{1,2}
Different Expression Pattern of TIM-3 and Galectin-9 Molecules by Peripheral and Peritoneal Lymphocytes in Women with and without Endometriosis
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A haptoglobin jelentősége az endometriosisos betegek megítélésében [The importance of haptoglobin in the assessment of patients with endometriosis]

Magyar Nőorvosok Lapja, közlésre elfogadott [Hungarian Gynecology Journal, accepted for publication]

Lectures and posters given on the topic of the thesis

Poster

Adenocarcinoma arising from adenomyosis, a case report

EBCOG (European Board & College of Obstetrics and Gynaecology) kongresszus, Torino, Italy, 2016

Lecture

Az endometriosis diagnosztikája [Diagnosis of endometriosis]

Magyar Kontinencia és Urogynecológiai Társaság Kongresszusa [Congress of the Hungarian Society of Continence and Urogynecology], Pécs, 2019

Endometriosisos betegek életminőségének javítása [Improving the quality of life of endometriosis patients]

Szülészeti-nőgyógyászati, gyermeknőgyógyászati pszichoszomatika, Oftex tanfolyam

[Obstetrics and gynecology, pediatric gynecology psychosomatics, Oftex course], Pécs, 2023

Scientific publications not on the subject of the dissertation

1.Papp Szilárd dr.¹ Varga Tamás dr.² Gőcze Péter dr.³ **Szegedi Sarolta dr.**⁴ Kovács Krisztina dr.⁵ Stefanovits Ágnes dr.⁶ Bárdos Nikoletta dr.⁷ Bohonyi Noémi dr.⁸ Koppán Miklós dr.⁹

Kiterjesztett regionális lymphadenectomia endometrium-carcinoma esetén: előnyök és hátrányok [Extended regional lymphadenectomy for endometrial carcinoma: advantages and disadvantages]

Magyar Nőorvosok Lapja [Hungarian Gynecology Journal] 82 : 6 pp. 305-309., 5 p. (2019)

2.Halvax László; **Szegedi Sarolta**; Rácz Sándor; Csermely Tamás; Vizer Miklós; Bódis József;

Spontán szülés corporalis longitudinalis császármetszés után. [Spontaneous birth after corporal longitudinal caesarean section.]

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